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ERRATA

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Page 380: First paragraph under heading "Conclusions," third line of paragraph, "37 per cent" should read "57 per cent."

Page 380: Third paragraph under heading "Conclusions," third line of paragraph, "39 per cent" should read "45 per cent."

PHYSIOLOGICAL STUDIES OF ACCESSORY AND STIMULATING FACTORS IN CERTAIN MEDIA

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INTRODUCTION AND PURPOSE

This investigation deals with the factors making for rapidity and efficiency in the process of cellulose decomposition. The present paper is concerned mainly with the influence of certain essential food substances.[1]

Work upon the growth-accessory or essential food substances and microbial nutrition begins with Pasteur's medium (Pasteur, 1859), and continues with Wildiers' introduction of "bios" (Wildiers, 1901), confirmed later by Williams (1919) and Bachman (1919). Other accessory substances have occupied the attention of workers, such as the factors of Thjötta and Avery (1921), while Miller and his associates (1924) are proceeding with the study of "bios."

Synthesis of essential food substances by bacteria is demonstrated by the work of Bottomley (1919), Hunter (1923) and Mockeridge (1924).

Stimulation of the physiological activities of the living cell through the agency of these substances has received a great deal of consideration (Schreiner and Skinner, 1912; Warburg, 1914; Bottomley, 1917; Warington, 1923; Gray and Chalmers, 1924; Puri, 1924; Cowdry, 1924; Meyerhof, 1924).

THE SELECTION OF AN ORGANISM

The organism used in this investigation, *Cellulomonas folia*,[2] was isolated by the author from decomposing leaves, and attacks cellulose vigorously with the formation of acid.

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[1] Other papers to follow.

[2] *C. folia*, a new species will be described elsewhere.

METHOD OF PROCEDURE

Some information regarding the rate of cellulose decomposition by *C. folia* can be obtained by measuring the hydrogen-ion concentration changes in the medium during the process of fermentation. For this purpose a medium was employed which would lend itself readily to the hydrogen-ion technique. This medium was a modification of McBeth's solution (McBeth, 1916), and proved suitable mainly because its "buffer index" was found to be negligible and because it will not by itself support the growth of *C. folia*. The composition of the medium is as follows:

K_2HPO_4	1 gram
$MgSO_4$	1 gram
Na_2CO_3	1 gram
$(NH_4)_2SO_4$	2 grams
H_2O (dist.)	1000 cc.

This nutrient solution was employed exclusively in my experiments. The figures given below show the fate of *C. folia* in this nutrient solution. Platings were made very four hours using nutrient agar (pH 7.6) upon which the organism grows readily.

HOURS	BACTERIAL NUMBERS	HOURS	BACTERIAL NUMBERS
Initial	35,000,000	24	50,000
4	6,000,000	28	20,000
8	2,500,000	32	10,000
12	1,000,000	36	5,000
16	500,000	40	5,000
20	100,000	44	1,000
		48	1,000

For the fermentation experiments cellulose was added to the nutrient solution. Increase in growth or physiological activity in this medium may be traced directly to the presence of cellulose.

EXPERIMENTAL

1. Influence of autoclaved and unautoclaved maple leaves upon the the growth of C. folia, and the Decomposition of Cellulose as measured by the Hydrogen-ion concentration

Experiment a. Hand-picked maple leaves, dried in the laboratory, were employed. Some were sterilized in the autoclave for twenty minutes at 15 pounds pressure in liter flasks containing the nutrient solution. Other portions were immersed in sixty per cent ethyl alcohol for forty-eight hours followed by copious washing with sterile water. The treatment with alcohol proved to be an efficient means of sterilization. The leaves remained sterile in about 60 per cent of the cases. To ascertain this point, the leaves in 2-gram quantities were incubated in liter flasks containing 200 cc. of nutrient solution. Incubation took place at 27° for forty-eight hours. The flasks which remained sterile received inoculations from a *mixed* culture composed of two cellulose-decomposing organisms. One of these was *C. folia*; the other, also a new species isolated from the decomposing leaves, has been called *Actinomyces colorata*. From time to time during the fermentations microscopic examinations were made of the medium for contaminating forms. In every case, however, there was no contamination.

TABLE 1

Effect of autoclaving as revealed by the activity of C. folia and Act. colorata upon leaves

DAYS	UNHEATED LEAVES (pH)	AUTOCLAVED LEAVES (pH)	CONTROL CHANGE OF pH IN MEDIUM
Initial	6.80	6.80	6.80
2	6.30	6.85	6.80
4	5.45	6.95	6.80
6	4.87	7.00	6.90
8	4.50	6.95	6.90
10	4.18	6.84	—
12	3.90	6.70	—
14	3.75	6.60	—
16	3.70	6.55	—
18	3.74	6.54	—
20	3.90	6.57	—
22	4.10	6.65	6.94
24	4.35	6.67	—
26	4.55	6.69	—
28	4.75	6.70	—
30	5.00	6.70	—
32	5.20	6.72	—
34	5.35	6.73	—
36	5.48	6.73	—
38	5.60	6.74	—
40	5.70	6.75	7.0

Determinations of the hydrogen-ion concentrations[3] produced by the apparent destruction of cellulose, as well as of other associated substances were made at frequent intervals in both series. In the control flasks, without the organisms, the pH change was very slight. The results are shown in table 1.

From the above experiment it becomes immediately apparent that there is a marked difference in the activity of the organisms upon the leaves in the two cases.

Experiment b. In order to establish the influence of this unheated sterile "leaves medium" as a favorable environment, *C. folia* was cultivated upon the medium for several weeks, making transfers every seven days. Maple leaves were employed because of their wide distribution in the vicinity. As before, care was taken to insure sterility by preliminary incubation and frequent microscopic examinations. For comparison the same organism was kept upon nutrient (beef-peptone) agar (pH 7.6). *C. folia* was cultivated upon this medium for nine months, making transfers every two weeks. During this time the organism became accustomed to nutrient agar. These two cultures, to be

TABLE 2

Changes in the H-ion concentration of medium during the decomposition of cellulose

DAYS	A (pH)	B (pH)	CONTROL (pH)
Initial	8.50	8.50	8.50
2	8.38	8.20	8.47
4	8.26	7.90	8.45
6	8.20	7.61	8.40
8	8.15	7.28	8.38
10	8.10	6.77	8.34
12	8.08	6.02	8.20
14	8.00	5.50	8.12
16	7.90	5.15	8.06
18	7.80	4.94	8.02
20	7.70	4.82	7.95
22	7.66	4.80	7.90
24	7.63	4.87	7.84
26	7.60	4.94	7.78
28	7.58	5.00	7.73

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[3] The electrometric method described by Itano was used. Jour. Bacteriol. (1923) **8**, 521.

referred to as "B" and "A" respectively, were used to inoculate liter Erlenmeyer flasks, each containing 200 cc. of the basic nutrient solution and three grams of cellulose in the form of raw cotton.[4] The flasks were incubated at 27°. The changes in hydrogen-ion concentration during the decomposition of the cellulose were determined. The results are given in table 2. The control flasks contained the nutrient solution without cellulose.

It is evident that some significant factor is operating in the untreated leaves, which exerts a considerable influence upon *C. folia.*

Experiment c. Subsequent investigation of the leaf compost further demonstrated the influence of this factor. The latter, though thermolabile, is readily obtained in the form of an aqueous extract by filtration of an aqueous suspension of the compost through a sterile porcelain filter. The filtrate was found to exert a marked influence upon the efficiency of cellulose-destroying microörganisms in laboratory experiments. A medium was prepared having the compost as a basis. This material had been autoclaved for fifty minutes at 15 pounds pressure. A sheet of English filter paper, 11 cm. in diameter and sterile, was pressed against the surface of the compost. The inoculum consisted of a twenty-four hour nutrient solution activated culture of *C. folia.* Two cubic centimeter portions of the filtrate were added to a series of flasks containing this medium. Controls were carried at the same time. In the presence of the filtrate complete destruction of the filter paper took place in four weeks, not a trace of the paper remaining. In the control flasks, without the filtrate, the decomposition was considerably slower and the filter paper was never wholly destroyed during the entire experiment which lasted three months.

2. The influence of vitamine B (?) upon the growth and physiological efficiency of C. folia

The preparation of vitamine employed was Vitamine-Harris[5] the highly-concentrated vitamine, water-soluble-B (fraction 11), described by Osborne and Wakeman (1919), which is prepared from fresh brewer's yeat. The preparation consists largely of the intracellular content of the yeast cell, and while it is free from native protein, not giving the color reactions for proteins, it does contain nitrogenous derivatives of the yeast proteins, such as amino acids.[6] The potency of this vitamine preparation has been determined by Bailey (1922) in a series of biological tests upon young albino rats.

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[4] Raw cotton obtained from the University of Arkansas; unginned and chemically untreated.

[5] Vitamine B (?), Yeast Vitamine, prepared by the Harris Laboratory, Tuckahoe, New York.

Itano (1923) obtained positive results in his investigation of the influence of vitamine upon *Azotobacter*. He used the same preparation of vitamine employed in this investigation and the one described above.

TABLE 3

Influence of vitamine B (?) upon the growth of C. folia

Expressed in millions

HOURS	CONTROL	1:10,000	1:100,000	1:1,000,000	1:10,000,000
6	20	20	20	20	20
12	20	25	75	25	20
18	20	32	100	25	20
24	20	41	117	24	20
30	16	54	130	23	18
36	12	74	139	22	15
42	8	115	126	21	12
48	6	180	90	20	10

A 1 per cent solution of the yeast vitamine powder, in which vitamine B is highly concentrated, was prepared, and from this a series of vitamine dilutions were set up. One cubic centimeter portions of these dilutions were added to tubes of nutrient solution to give the following dilutions: 1:1000, 1:10,000, 1:100,000 and 1:1,000,000. The inoculum consisted of 0.1 cc. of a twenty-four nutrient solution culture of *C. folia*.

Preliminary tests based upon visual observation of turbidity showed positive results only in the vitamine concentrations 1:1000 and 1:10,000 after twenty-four hours' incubation at 30°.

Table 3 shows that vitamine dilutions 1:10,000 and 1:100,000 exert a marked stimulating influence upon the growth of *C. folia*. Platings were made every six hours, using nutrient agar pH 7.6.

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[6] Yeast Vitamine, Harris Laboratory Bul. No. 14.

Although the increase in the rate of cellulose decomposition is apparent when vitamine is added to the nutrient medium, the hydrogen-ion technique was found to be inapplicable on account of the highly buffered character of the resulting medium. The fermentation flasks were set up as in experiment b using raw cotton, and suitable concentrations of vitamine in conjunction with the nutrient solution. Microscopical examination of the cotton fibers showed evidence of more active destruction of cellulose than in the control flasks containing no vitamine. Stimulation in growth and physiological efficiency was apparent in vitamine dilutions 1:10,000 and 1:100,000. These results are in accord with those obtained by Itano (1923) in testing the influence of vitamine upon *Azotobacter*.

3. The influence of extracts from seeds and seedlings upon the growth and physiological efficiency of C. folia

Extracts were prepared from one representative of each of the families, *Leguminosae*, *Gramineae*, and *Polygonaceae*. Alfalfa, barley and buckwheat seeds were used, and extracts prepared from the seeds before and after germination; also from the seedlings.[7]

a. Ungerminated seeds. A weighed quantity of seeds was sterilized for two minutes in mercuric chloride (1:500), and washed thoroughly in sterile water. The seeds were ground in a mortar, and one per cent aqueous extracts made. The reactions of the latter were adjusted to pH 4.6, and each extract was boiled for ten minutes.[8]

b. Germinated seeds. The seeds were washed and sterilized as described above, then transferred under aseptic conditions to a moistened filter paper in sterile petri dishes. After forty-eight hours' incubation at 27°, during which time most of the seeds germinated, a number of the seeds were transferred under aseptic conditions to sterile weighing bottles, the weights of which had been previously ascertained. The seeds were dried over sulphuric acid until they attained a constant weight, then ground to a powder in a mortar and one per cent aqueous extracts prepared. The reactions of the latter were adjusted as before, followed by boiling for ten minutes.

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[7] Method used by Thjøtta and Avery, Jour. Exper. Med (1921) 34; 97.

[8] The extracts in this experiment were subject to a short autoclaving, 15 pounds for ten minutes, instead of boiling over free flame as was done in the case of the other extracts prepared. The work of Wildiers and others show "Bios" to be unaffected by boiling in an acid environment, although strong acid destroys it.

TABLE 4

Influence of the extracts upon the growth of Cellulomonas folia

HOURS	ALFALFA, BARLEY AND BUCKWHEAT EXTRACTS (1:10 DILUTION)—TOTAL DILUTION (1:1000) EXPRESSED IN MILLIONS								
	Seeds	Alfalfa germinated seeds	Seedlings	Seeds	Barley germinated seeds	Seedlings	Seeds	Buckwheat germinated seeds	Seedlings
Initial	35	35	35	35	35	35	35	35	35
4	3	6	5	5	9	16	20	26	24
8	8	24	8	12	16	31	30	38	30
12	29	60	20	24	14	53	44	60	42
16	49	92	34	37	17	74	57	84	54
20	70	127	47	50	26	98	71	106	66
24	92	145	59	63	43	120	85	130	77
28	113	150	71	71	86	132	99	146	94
32	129	153	82	79	141	146	113	162	111
36	134	152	88	87	194	159	123	180	128
40	133	147	91	96	250	172	129	196	145
44	127	136	92	105	302	185	132	212	162
48	78	120	90	114	360	200	132	230	180

c. Seedlings. The seeds were sown in pots containing sandy loam and grown in the greenhouse under suitable temperature and moisture conditions for a week or ten days.

	ALFALFA	BARLEY	BUCKWHEAT
Length of time grown, days	10	7	10
Length of shoot, cm	1–1.5	2–3.5	2–3.5

The entire seedling was removed from the pot and washed free of soil particles. The seedlings were sterilized as in the case of the seeds, washed and dried. One per cent suspensions were prepared as before. Table 4 shows the influence of these extracts upon the growth of *C. folia*.

Table 5 shows the increase in bacterial numbers under the influence of the extracts. The figures recorded apply to an extract dilution of 1:10. Other dilutions were employed, namely 1:1000, 1:100,000 and 1:1,000,000, but the acceleration in growth became much less apparent.

The stimulating effects of the extracts upon the physiological efficiency of *C. folia* were readily determined by the H-ion method.

TABLE 5

Influence of alfalfa, barley and buckwheat extracts upon the cellulose-decomposing ability of C. folia

DAYS	CONTROL (pH)	ALFALFA EXTRACTS (pH)	BARLEY EXTRACTS (pH)	BUCKWHEAT EXTRACTS (pH)
Initial	8.40	8.40	8.40	8.40
2	8.37	8.14	8.30	8.02
4	8.33	8.02	8.20	7.88
6	8.27	8.00	8.10	7.80
8	8.21	8.00	7.98	7.70
10	8.16	7.94	7.84	7.62
12	8.10	7.86	7.67	7.54
14	8.04	7.78	7.54	7.46
16	7.98	7.68	7.48	7.40
18	7.92	7.57	7.44	7.30
20	7.86	7.46	7.40	7.22
22	7.81	7.36	7.36	7.12
24	7.75	7.24	7.28	7.01
26	7.70	7.10	7.18	6.90
28	7.64	6.97	7.09	6.79
30	7.58	6.84	7.00	6.66
32	7.53	6.71	6.91	6.57
34	7.50	6.58	6.82	6.48
36	7.42	6.44	6.72	6.40

The same general procedure was followed as in previous experiments. Raw cotton was used as a source of true cellulose, and 0.05 per cent of the active principle of the extracts was added to the nutrient medium. The control represents the action of the organism upon cellulose without the addition of the extracts. The action of *C. folia* upon nutrient solution containing the same concentration of extract as in the other flasks only without cellulose, was determined. No appreciable change in the H-ion concentration of the medium was observed over the normal changes exhibited by the nutrient solution itself as recorded in the "control" column, table 2.

The extracts exerted a marked stimulative influence upon the growth and physiological efficiency of *C. folia*. In general, the extracts from the germinated seeds exhibited a greater stimulative effect than the extracts from the ungerminated seeds and seedlings as far as growth was concerned.

TENTATIVE CONCLUSIONS

1. The physiological efficiency of cellulose-decomposing organisms is markedly influenced by the composition of the medium in which they have been cultivated.
2. The method of sterilization by autoclaving effects sufficient change in the composition of maple leaves to influence considerably the action of cellulose-destroying organisms upon them.
3. The essential food factor represented by vitamine B (?) exerts a stimulating effect upon the growth and physiological efficiency of *C. folia*.
4. The essential food factor contained in extracts prepared from the seeds and seedlings of alfalfa, barley and buckwheat, exhibits a marked stimulative influence upon the growth and physiological efficiency of the organism.
5. The determination of hydrogen-ion concentration changes during cellulose decomposition is a legitimate criterion of the physiological efficiency of *C. folia*.

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THE EFFECT OF SURFACE TENSION UPON THE GROWTH OF LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS BULGARICUS

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The attention of many workers in the field of bacteriology has been attracted by the interesting problems presented by the Gram-positive bacteria comprising the genus *Lactobacillus*. Stimulated by the theory and subsequent investigation of Metchnikoff and more recently by the work of Rettger and Cheplin, the greater part of this work has been devoted to the two most generally known members of the genus, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*.

Not the least interesting of the problems presented by this group of bacteria is the cultural differentiation between *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, complicated as it is by their close similarity, both morphologically and culturally. Indeed this smilarity is so close as to have led Rodella (1901) and Heineman and Hefferan (1909) to conclude that they were identical. Rahe (1914) proposed a differentiation based on the fermentation of maltose. Kulp and Rettger (1924), in an exhaustive comparative study of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, as part of their conclusions state: "The action upon maltose, sucrose and levulose appears to furnish a valuable means of separating strains of these two groups. . . . ."[1] Unpublished work done in these laboratories on the sugar fermentations of these organisms has however given variable results.

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[1] An excellent review of the literature is contained in this paper.

It would seem then, that a differentiation of these two types of bacteria based on sugar fermentations alone is not absolutely infallible, and a method of distinguishing culturally between the two closely related organisms is most desirable.

A review of the voluminous literature on the implantation of the aciduric bacteria in the intestinal tract seems to justify the conclusion that *Lactobacillus bulgaricus* can not be implanted in the intestinal tract while the implantation of *Lactobacillus acidophilus* is more or less easily accomplished. That the bile salts may play some part in this natural selection suggested itself; and as they are surface tension depressants, separation on this basis was attempted.

EXPERIMENTAL

Fifteen strains each of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* were employed in this work. Seven strains of each were obtained from authentic sources and comprise the majority of the strains most frequently reported upon in the literature. The remaining eight strains of each constitute the representative strains of these organisms in a collection of this genus maintained in these laboratories, and include a strain of *Lactobacillus bulgaricus* used by Metchnikoff in his work on this organism and a strain of *Lactobacillus acidophilus* obtained from the collection of the American Museum of Natural History in 1919.

The media used are designated as medium X and medium Y. Medium X was made up of 1 per cent yeast, 1 per cent pepton, 1 per cent lactose, 1 per cent beef extract, and 0.5 cc. of a 5 per cent alcoholic solution of brom cresol purple per liter, as an indicator. In medium Y, 1 cc. of skimmed milk per 100 cc. of medium was substituted for the beef extract of medium X. In each experiment the medium was divided into four equal parts, one part being tubed as control while to each of the three remaining portions varying amounts of the surface tension depressant were added before tubing. After sterilization the hydrogen ion concentration of each medium was determined electrometrically, and the surface tension was determined by the weight-drop method. The sodium recinolate used in these experiments was prepared in these laboratories, and the sodium taurocholate and sodium glycocholate were commercial products.

Inoculations were made into the test media from mature milk cultures with a platinum loop; incubation was at 38°C. The slightest perceptible change in the color of the medium after an incubation of seven days was accepted as an indication of growth and so recorded.

Figures 1 and 2 illustrate the effect of reduced surface tension upon the growth of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* when grown in the same medium. Sodium recinolate was used as the depressant. The figures have been so drawn as to illustrate the number of strains employed in the experiment and the number of strains of *Lactobacillus bulgaricus* inhibited in each of the depressed media employed. As previously stated, a perceptible change in the color of the medium was recorded as growth. The surface tension of the control media varied from 52 to 56 dynes, but all controls are represented in the graphs as having a surface tension of 56 dynes. The ends of the graphs representing the growth of *Lactobacillus acidophilus* are left broken, for in every case all the strains of *Lactobacillus acidophilus* made sufficient growth to change completely the color of the medium to yellow; therefore the figures cited do not represent the lowest surface tension at which these organisms are able to grow.

It will be seen in figures 1 and 2 that none of the strains of *Lactobacillus bulgaricus* showed evidence of growth after seven days incubation at 38°C., when the medium employed was depressed with sodium recinolate to a surface tension of less than 40 dynes, and that most of the strains were inhibited well above this value; while all the strains of *Lactobacillus acidophilus* were able to grow when the medium was depressed to a surface tension as low as 36 dynes. It would appear from the figures that the pH of the medium influences to some extent the ability of these organisms to grow in a medium of reduced surface tension. An interesting point, not brought out in these figures, is that even in the medium depressed to 36 dynes all but two of the strains of *Lactobacillus acidophilus* had produced sufficient acid in from

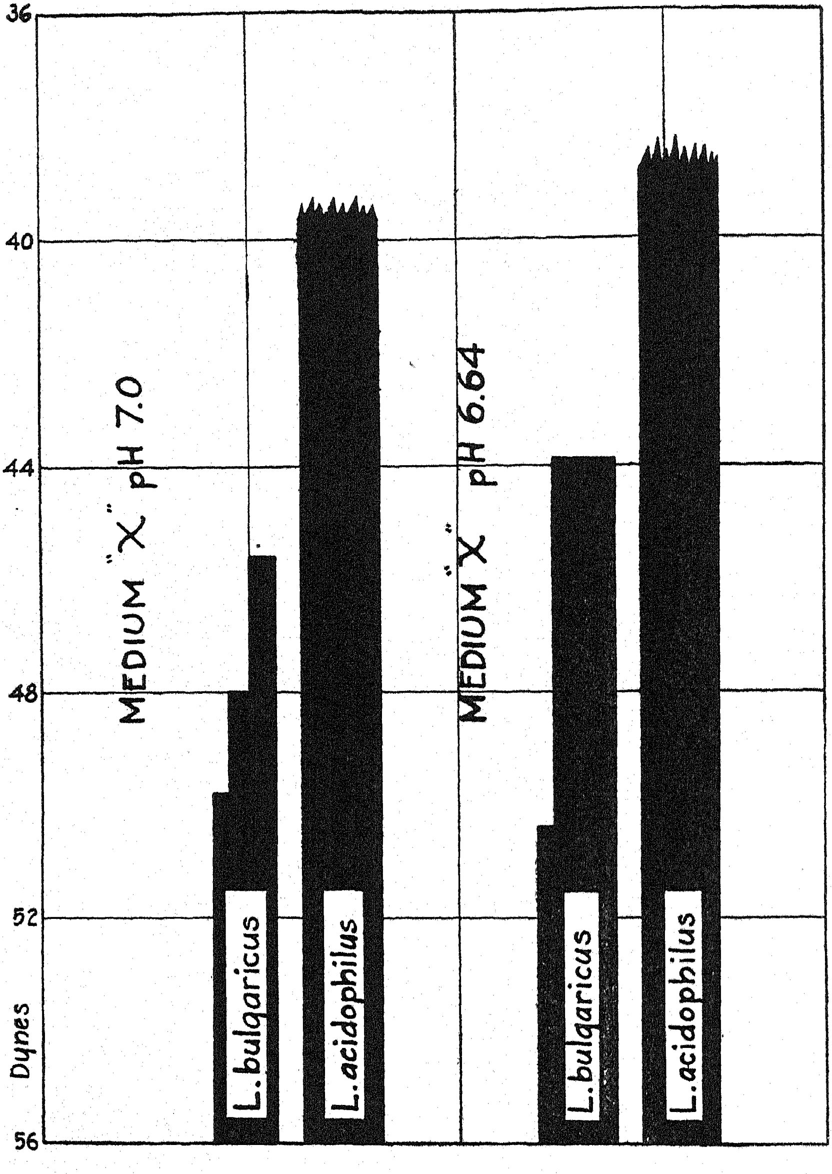
36
40
44
48
52
56
Dynes
MEDIUM "X" pH 7.0
MEDIUM "X" pH 6.64
L. bulgaricus
L. acidophilus
L. bulgaricus
L. acidophilus

Fig. 1

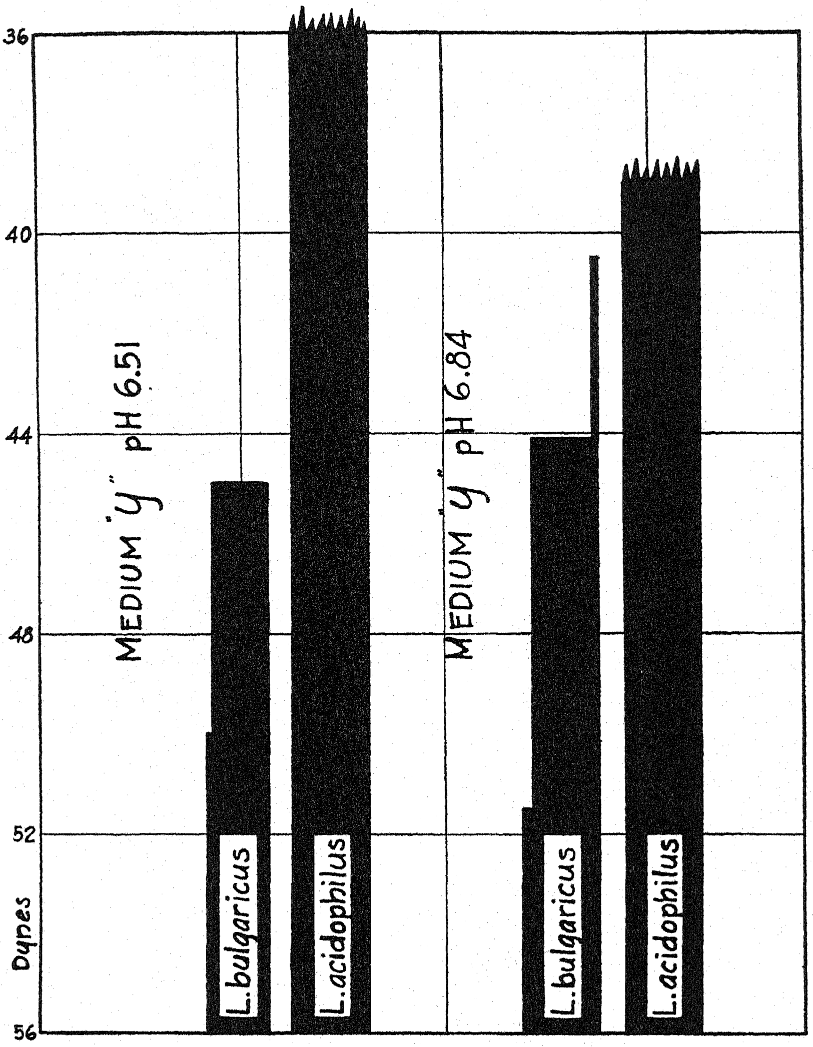
36
40
44
48
52
56
Dynes
MEDIUM "Y" pH 6.51
L. bulgaricus
L. acidophilus
MEDIUM "Y" pH 6.84
L. bulgaricus
L. acidophilus

Fig. 2

twenty-four to forty hours completely to change the color of the medium to yellow, the two tardy strains requiring fifty-four and ninety hours.

Sodium taurocholate was employed in one experiment, using medium Y. The lowest surface tension obtained was 41 dynes at a pH of 6.5. Six strains of *Lactobacillus bulgaricus* showed growth in the medium of the lowest surface tension after six days incubation, but nine strains were inhibited. However, all but one of the strains of *Lactobacillus acidophilus* had completely changed the color of the medium to yellow in from fifteen to twenty-four hours, the tardy strain requiring forty hours.

One experiment in which sodium glycocholate was used as a depressant in medium Y gave inconsistent results.

The inconsistent results obtained with sodium taurocholate and sodium glycocholate may well be due to the large amounts of these salts necessary to obtain a medium of low surface tension.

CONCLUSIONS

1. In the media employed in which sodium recinolate was used as the depressant, *Lactobacillus bulgaricus* is inhibited at a surface tension lower than 40 dynes while *Lactobacillus acidophilus* exhibits good growth in the same media depressed to 36 dynes.
2. That surface tension may be a factor in the implantation of these organisms seems a plausible assumption.

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SPIRILLUM VIRGINIANUM NOV. SPEC.[1]

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INTRODUCTION

Spiral microörganisms living in stagnant water and in infusions made from decaying hay, leaves, mud, etc., have been described by the early investigators on "infusion animalcula." It was not until the latter half of the seventeenth century that the Jesuit, Kircher, in 1659, and the Dutch linen-draper, van Leeuwenhoek, in 1675, actually saw and described living things too small to be seen with the naked eye. During the century following the work of these pioneers, the efforts of investigators were chiefly devoted to the more exact morphological description of some of the forms of unicellular life, already known. From that time to the present many species of spiral microörganisms, pathogenic and saprophytic have been described.

The term spirilla was originally used to describe spiral, cork screw organisms regardless of whether they were vibrios, spirilla or spirochaetes. The definition of spirilla as accepted at present is as follows: The microörganisms belonging to the genus Spirillum are free-living or parasitic, non-flexuous, spirals of various thicknesses, length and pitch of spiral, curved in three planes, forming either long spirals or a portion of a turn, non-motile or motile by means of flagella at one or both poles. Reproduction is by transverse division. The difference between spirillum and spirochaete is that the former possesses a rigid, non-flexuous cell body, while the latter has a flexuous cell body and no true flagella.

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[1] Dissertation submitted in partial fulfilment of the requirement for the degree of Doctor of Science in Hygiene in The School of Hygiene and Public Health, the Johns Hopkins University.

The difference between spirillum and vibrio is that the former curves in three planes and the latter curves in only two.

This investigation was devised to study the spirilla according to the standard methods for studying pure cultures of bacteria as follows:

1. Experiments on the cultivation of spirilla.
2. The separation of the spirilla from the other bacteria associated with them.
3. The morphological study of pure cultures of spirilla.
4. The physiological study of pure cultures of spirilla.
5. Their identification and classification.

REVIEW OF LITERATURE

The term "Spirillum" originated with Ehrenberg (1838) when he described *Spirillum undula, Spirillum volutans*, and *Spirillum tenue*. He defined spirillum as follows: "animal belonging to the family Vibrios, multiplication takes place by spontaneous imperfect (oblique?) division, forming tortuous spiral, rigid and cylindrical chains (filaments)." Ehrenberg's *Spirillum undula* was probably the same organism which Moeller (1786) had designated *Vibrio undula.*

No reports of researches on environmental spirilla seem to have been made from 1838 until Sorokin (1886) discovered a branching spiral organism, *Spirillum endoparagicum* in the white exudate from an old hollow black poplar tree. The spirillum seemed to have existed in pure culture but no attempt to cultivate it was reported.

In the following year Esmarch (1887) discovered *Spirillum rubrum* in pure culture in the pale red, dry, crumbling remains from a putrified mouse that had fallen in a basin of rain water.

During April Kutscher (1895) reported his observation on the vibrios and spirilla found in the juice obtained from decaying manure and succeeded in isolating *Spirillum tenue, Spirillum undula*, and *Spirillum volutans*. During October of the same year Kutscher reported his further observations on what he called *Spirillum undula.* In order to check up his conclusion Kutscher sent a culture of his spirillum to Professor Zettnow who observed that this organism was smaller than what was considered at that time *Spirillum undula* (Cohn) and accordingly suggested the name *Spirillum undula-minus* to distinguish it from *Spirillum undula* (Cohn) later called *Spirillum undula-majus*.

Among other forms Swellengrebel (1909) described *Spirillum volutans* and *Spirillum parvum* as possessing "finely honeycombed protoplasm with chromatin substance distributed through the cytoplasm in transverse and zigzag bands."

Fuhrmann (1909) described in great detail the origin of the flagellum in *Spirillum volutans* as "arising from the external membrane and meeting the cytoplasm in immediate union."

Hölling (1911) distinguished between "Spirochaeta" and "Spirillum" by finding rigid, non-flexuous cell bodies in the spirilla, in contradistinction to the flexuous cell bodies of the spirochaetes.

EXPERIMENTAL

a. Methods of cultivation

The initial culture studied in the present investigation was obtained from the mud that had adhered to the outside of an oyster shell. This mud was emulsified with tap water and incubated in the ice box for twenty-four hours when a hanging drop preparation revealed rapidly motile spiral microörganisms in great numbers swarming in the emulsion. Many unsuccessful attempts were made to cultivate this spirillum on the various laboratory media and in combinations of Ringer's solution and sea water. A combination of 75 per cent sea water and 25 per cent egg cube medium[2] proved a good medium for growing the first cultures of this spirillum, although it supported life only a few days. Many other kinds of media and combinations of various media were tried and eventually one other gave good results. It was prepared as follows:

200 cc. physiological salt solution
50 cc. egg cube medium
30 cc. liquid 2 per cent agar in distilled water

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[2] Egg cube medium is prepared by putting a small cube of hard boiled egg white in 10 cc. beef extract broth and sterilizing it in the autoclave.

In this medium the spirillum grew well but it appeared smaller than the spirillum originally seen.

Later a 0.7 per cent semisolid medium was obtained on which these spirilla grew luxuriantly. They grew very scantily on the surface, but in a stab made in the medium they produced an abundant, filiform growth all along the line of puncture. This medium was prepared as follows:

40 cc. liquid 2 per cent agar in distilled water
68 cc. distilled water
20 cc. sterile egg cube medium

After adaptation to laboratory conditions these spirilla were studied in pure cultures obtained by plating on beef extract agar to which egg cube medium was added in the following proportions:

75 cc. liquid beef extract agar
25 cc. egg cube medium

On this medium the spirilla produced small, dew-drop, convex, glistening, slightly opaque colonies. Discrete colonies were fished out and transferred by the stab method to 0.7 per cent semisolid beef extract agar. The procedures of plating, fishing out the colonies and transferring them into semisolid medium were repeated until pure cultures of spirilla were obtained. In the course of the experiment a 0.7 per cent beef extract agar (without the egg cube medium) was used and on it the spirilla grew luxuriantly.

b. Methods of staining

The morphological study of these spirilla was carried on with pure cultures grown on the semisolid medium mentioned above for forty-eight hours at 18°C. During the preliminary experiments many staining methods were tried. Neither Heidenhain's nor Wright's iron haematoxylin yielded satisfactory results. Giemsa's solution was not satisfactory in studying the spirilla because of the amount of time it required for staining the preparations and because the details of morphology were not accentuated. Noguchi's (1921) method for staining cristispiras gave fair results, but the spirilla appeared shrunken. Loeffler's methylene blue stained them lightly. By Gram's method they stained red. Carbol fuchsin stained them fairly well, while Ziehl-Neelsen's method for staining acid-fast bacteria rendered the spirilla distorted, swollen, and hardly recognizable. With Benigetti's method for staining flagella as given by Besson the spirilla appeared very large and swollen. Loeffler's flagella stain did not stain the flagella of these spirilla. Fontana's method for staining spirochaetes yielded very poor results. It was found that if heat was applied during the process of staining, the spirilla were altered to such an extent that the study of their morphological characteristics was impossible. A combination of Fontana's solution no. 1, with Sterling's anilin gentian violet-carbol fuchsin proved the most satisfactory stain. Its application was carried out as follows:

1. The air-dried film was covered with Fontana's solution no. 1 (acetic acid, 1 part; formalin, 2 parts; distilled water, 100 parts) for two minutes and washed in water
2. The film was then immersed from two to five minutes in a stain prepared as follows:
    - *a.* 2 cc. Sterling's anilin gentian violet
    - *b.* 2 cc. of 10 per cent basic carbol fuchsin
    - *c.* 60 cc. distilled water

This method was effective in staining the flagella. In routine work the Fontana's solution was omitted.

For the study of internal structure moist films were fixed in freshly mixed Zenker's fluid and stained by the gentian violet-carbol fuchsin method mentioned above. Vital staining with malachite green, methyl green, crystal violet, neutral red, and safranin was used to advantage in the study of internal morphology.

Dark-field illumination proved especially useful in studying the refractive granules and the position of the flagella.

Air-dried films were prepared and stained as already described. They show the spirilla stretched out rather than spiral (plate 5).

Figure 10 suggests, in a small degree, the normal, living spirillum. Plate 5 also shows the type and position of the flagella. The spirilla were stained solidly when they were kept in the dye longer than two minutes, but exhibited a barred appearance when they were stained for one minute (fig. 7, plate 2; figs. 5 and 6, plate 4). The appearance of the barred staining was perhaps due to the contraction of the cytoplasm in transverse bars during the drying and fixing processes.

The internal structure of the spirilla was studied in moist films prepared as follows: Albumin fixative was spread very thinly over a perfectly clean glass slide, in order to hold the spirilla and to prevent them from being washed off during staining and rinsing. A loopful of the culture was spread over the albumin fixative and exposed to osmic acid vapor for one minute. The glass slide with the film on it was then immersed in freshly mixed Zenker's solution for fifteen minutes, gently rinsed with water, stained with very dilute (0.1 per cent) gentian violet-carbol fuchsin for one minute, rinsed in water, dried, and examined under the oil immersion objective. It is essential to have the dye very weak if a delicate, distinct staining of the chromatin material is desired. Most of the spirilla were washed off during this process, but enough remained to permit study.

This method of staining apparently did not cause the cytoplasm to segregate in the form of transverse bars, although some element in the cytoplasm, chromatin material probably, was stained heavily as is shown by figures 4, 8, 9, 10, plate 2. The deeply staining material was distributed as small dots along the cell wall, as is shown in figure 4, plate 2, although in figure 9 most of the deeply staining material appears at the poles. The spiral threads running longitudinally through the cytoplasm were the most noticeable elements in the internal structure, although their accurate tracing was impossible (figs. 8 and 10, plate 2). In specimens that were almost straightened out the spiral threads could be followed definitely (fig. 9, plate 2). It should be noted that only one spiral thread is represented in figure 8, while two are represented in figure 9, plate 2. The spirillum represented by figure 10 was still motile when the drawing was made.

c. Vital Staining

Further study of the internal morphology was made by means of staining according to the method recommended by A. B. Lee. Methyl green, malachite green, and crystal violet were used to stain the chromatin material, while neutral red and safranin were employed to stain the plasma.

A 1 per cent aqueous solution was prepared from each of the five stains and filtered. The preparations were made by mixing one loopful of the culture and one loopful of the dye directly on the slide; a cover slip was placed over the film which was examined immediately under the high power, then under the oil immersion objective. Unfortunately, the spirilla died as soon as they came in contact with the dye, so the process of infiltration of the stain into the living organism was not observed.

Methyl green showed a marked affinity for the chromatin material, as is shown by the deeply stained portions in the cytoplasm (figs. 1 to 7, plate 4). The chromatin material was not distributed alike in all the organisms. Figure 2, plate 4, shows a large chromatin granule that appeared like a nucleus, but no similar observations were made on other specimens. Figures 4 and 7, plate 4, show the spiral thread running longitudinally through the cytoplasm, while the chromatin was distributed along the cell membrane at the points of contact between the cell wall and the spiral threads.

It was found that a 1 per cent aqueous solution of malachite green was too strong for vital staining so a 0.25 per cent solution was prepared which was found satisfactory. This dye showed a marked affinity for the chromatin in the spirilla, as is shown in figures 8, 10, 11, 12, plate 4

The spiral threads and the chromatin granules were plain in each specimen except that shown in figure 10, in which the chromatin granules appeared surrounded by thread-like septa. A lateral view of the spirillum was best suited for the study of the internal structure.

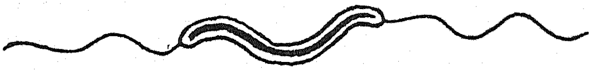
Crystal violet also proved effective in the staining of the chromatin material, as is shown by figure 9, plate 4. The dye seemed to stain the plasma as well as the chromatin.

Neutral red stained the chromatin fairly well, although it was used primarily to stain the plasma. Figures 13 and 14 show the spiral threads, but they are more pronounced in figure 13 than they are in figure 14.

Safranin showed the least affinity for the chromatin, while its affinity for the cytoplasm was strong, although the spiral threads were also seen (fig. 15, plate 4).

d. Morphology

This microörganism has a spiral, non-flexuous cell body consisting of ½ to 3 complete turns in young cultures, while in old cultures individuals are found which possess 7 turns. It is 3 to 11 micra long and 0.6 to 0.9 micron wide. The spiral amplitude varies from 4 to 5.6 micra and the width of the spiral is 1.2 micron. The contours of the cell body are parallel and the ends are rounded. It possesses a single, spiral flagellum at each pole, of the same spiral amplitude and width of spiral as the cell body. In fully grown individuals the flagellum is shorter than the cell body, but in young individuals the flagellum is longer. The spirilla appear barred when they are stained lightly, while they appear solid when they are overstained (plate 5). The morphology of the individual spirillum varies from a short comma to individuals possessing several turns. This variation is especially common in young vigorous cultures. In older, less vigorous cultures, longer spirilla are more numerous.



TEXT-FIG. 1. SHOWING THE RELATIVE POSITION OF THE AXIAL FILAMENT AND THE FLAGELLUM DURING MOTILITY

Observations upon the living Spirilla as seen under the dark field illumination. The motility of these spirilla is so rapid that no accurate observations are possible except as their movements become retarded. Motility is brought about by a typical corkscrew movement and a boring through the medium as the spirillum rotates on its long axis. The organisms possess no "anterior-posterior" polarity, but move in either direction with equal rapidity and facility. They move in one direction and then suddenly dart in the opposite direction. The effect of the flagella on the motility of the spirillum is clearly observed in slowly moving organisms. Both flagella are stretched out, as shown in text-figure 1. The forward movement of the spirillum is caused by the spiral rotation and circular switchings of the "posterior" flagellum, which appears to impart the rotating movement to the cell. In some instances the "anterior" flagellum was observed folded along the cell body during motility (text-fig. 5).

Further observations showed that the average spirillum possesses a single refractive flagellum at each pole, while the young (short) spirillum possesses a single flagellum at one pole. The flagella in the average spirillum are slightly longer than the cell body, whereas the flagellum in the young (short) spirillum is twice as long. The origin of the flagella appeared to be in the cell membrane, since no refractive material was observed in the cytoplasm opposite the "base" of the flagellum. The cell membrane and the flagellum refract the light with the same degree of intensity as is indicated by the dark areas represented by these organs. Figures 3, 5, 7, 9, 10, 14, and 15, plate 3, show that the flagella arise from the cell wall and not from the cytoplasm. The cell membrane (white area) appeared to be devoid of cytoplasm, since it did not take the stain (figs. 3, 13, 14, 15, plate 3).

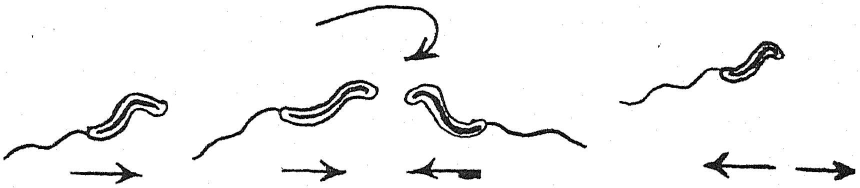


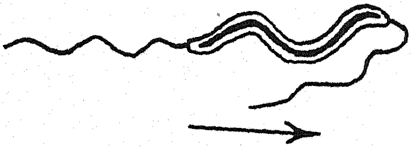
FIG. 2 FIG. 3 FIG. 4

The arrow indicates the direction of the movement

The short spirillum, possessing one flagellum, during forward movement has the flagellum extended "posteriorly" (text-fig. 2), but in reversing its direction the spirillum makes a quick "about face" and moves in the opposite direction (text-fig. 3). In other instances the direction of movement is reversed after the spirillum comes to a dead stop; it then darts in the opposite direction without turning about (text-fig. 4).

Observations under dark field illumination showed the spirilla to possess a refractive axial filament, running longitudinally through the cytoplasm, but not extending to the tip of the poles (text-figs. 1 and 5).

The area between the axial filament and the cell wall is translucent and at times very delicate, thread-like processes are observed extending from the axis to the cell membrane. Great care was exercised to determine whether or not these thread-like processes are the same as the spiral threads that were observed in plates 2 and 4. Their identity is not established because the thread-like processes are very indistinct and it is impossible to trace them accurately under the dark field illumination.



TEXT-FIG. 5. SHOWING THE RELATIVE POSITION OF THE FLAGELLA DURING MOTILITY

The arrow indicates the direction of the motion

Multiplication. A loopful of a vigorous culture of spirilla grown on semisolid medium for forty-eight hours at 18°C. was mixed with a loopful of liquefied gelatin medium. The preparation was made directly on the glass slide, a cover slip was laid over the film, sealed with vaseline, and the preparation examined under the high power objective. The rapid motility of the spirilla was retarded by the solidifying gelatin, and extensive observations on the living specimens were made possible. Long and short spirilla were observed, but the organisms of average length appeared to divide most frequently in the following manner:

The spirillum that is approaching the state of cell division becomes very sluggish. At this time a faint transverse line appears in the middle, in some organisms, while in others it is a little away from the middle (fig. 2, plate 1). As this transverse line becomes more pronounced the cytoplasm gradually separates and contracts away from it, toward the poles, leaving a transparent area on each side of this transverse line (figs. 3 and 4, plate 1). At this time a vibrating movement of the middle portion of the dividing spirillum is seen, as is indicated by the dotted lines in figure 3, plate 1. After a few moments the vibration ceases, but is followed by oscillating movements of the ends which swing like a pendulum, with the fixed point at the line of division, as is indicated by the dotted lines in figure 4, plate 1. During the oscillating movements the cell membrane begins to constrict at the transverse line, and gradually the constriction is completed (figs. 5 and 6, plate 1).

The new daughter cells remain attached to each other for some thirty minutes, at the same time lashing violently in a hinge-like fashion, each struggling to detach itself from the other. During motility the "posterior" cell causes the forward movement, while the other hangs on quiescent. When the direction of motion is reversed the quiescent spirillum becomes motile, while the other becomes immotile. Finally the organisms separate.

Involution forms. The figures shown in plate 3 represent the morphological types that were found in cultures one month old, stained as described on page 24. The characteristic contraction of the cytoplasm toward the poles is represented in figures 2, 3, 5, 9, 11, 13, and 15, plate 3. "Coccoid bodies" were also frequently observed (fig. 4, plate 3). Specimens as shown in figure 4, plate 2, figure 4, plate 3, figure 12, plate 5, are typical of "coccoid bodies" as they are found in old cultures of these spirilla. Some "coccoid bodies" possess flagella while others do not. Segregation of the cytoplasm in definite, heavily stained areas, suggests an analogy to spore formation in bacteria.

What have been called "budding" spirilla were found frequently in old cultures (figs. 1, 2 and 3, plate 2). Figure 2 was stained for flagella, and it is clear that each pole (bud) possesses a flagellum. On one occasion a budding spirillum was observed in a hanging drop preparation, but its motility was very sluggish.

The exact meaning of these involution forms is not clear. Further observations may be helpful in clearing up the life-cycle of the spirilla and of the spirochaetes.

e. Physiology

It was found that these spirilla were able to grow on the usual laboratory media after the organisms had been grown on the special semisolid medium for 40 generations and had become accustomed to artificial cultivation. This adaptation to laboratory conditions made it possible to study the cultural reactions of 26 pure cultures of spirilla on the following media: beef extract broth, lead acetate, egg cube medium, agar slant, nitrate solution, gelatin, lactose broth, mannitol infusion broth, glycerol broth, dulcitol broth, sucrose broth, dextrine broth, glucose broth, Uschinsky's protein-free medium. The media were adjusted to the optimum pH 8.2 to 8.4 before sterilization.

The inoculation of the media was made by introducing three drops into each tube from a four-day culture grown either on beef extract broth or on egg cube medium. A heavy inoculum was necessary for obtaining good growth. All the cultures were incubated in the ice box and daily reading of the cultural reactions were made for seven days. The cultures were kept for one month and during this time frequent observations were made.

The cultural characteristics of these spirilla can best be studied in a tabular form. Analysis of table 1 which gives the properties of *Spirillum volutans*, *Spirillum undula*, and of this new species shows that the points of similarity among these spirilla are found in their inability to produce indol and their inability to reduce the nitrates. The colonies produced on gelatin plates by *Spirillum volutans* and by the species described in this investigation are similar, while their growths in gelatin stab differ. The former produces porcelain-white, crumpled surface growth, with slight growth in the stab and slow liquefaction. *Spirillum virginianum* grows very scantily on the surface, but produces luxuriant, filiform, ivory-white growth in the stab without liquefaction. This species produces cloudiness (no flocculation) in broth, while the others produced turbidity. This species is Gram-negative, while the others are Gram-positive. The habitat of *Spirillum volutans* is stagnant water, and that of *Spirillum undula* is putrid and stagnant water, while this new species inhabits the muddy bottom of brackish water. The optimum temperature for *Spirillum volutans* is 20°C., for *Spirillum undula* 25°C., and for *Spirillum*

TABLE 1

Cultural characteristics of Spirillum volutans, Spirillum undula, and Spirillum virginianum

MEDIUM	SPIRILLUM VOLUTANS (BERGY*)	SPIRILLUM UNDULA (BERGY*)	SPIRILLUM VIRGINIANUM
Gelatin colonies.	Gray, smooth, entire glistening	Circular, granular, greenish-yellow	Entire, convex, circular, moist, colorless
Gelatin stab.....	Porcelain-white, crumpled, surface growth, slight growth in stab, slow liquefaction	White, rugous, surface growth, no liquefaction	Growth along entire stab, no liquefaction
Agar colonies....			Dew-drop, convex, entire moist, colorless
Agar slant.......			Dew-drop, isolated
Broth...........	Turbid	Turbid	Cloudy, seldom turbid
Litmus milk.....	Unchanged to slight alkalinity		No growth
Potato..........	Dry brown streak		No growth
Indol............	Negative	Negative	Negative
Nitrates.........	Not reduced	Not reduced	Not reduced
Gas in sugars....			None
Acid in sugars...			None
Uschinsky's protein-free medium.......			Abundant growth
Loeffler's coagulated serum..			Convex, dew-drop isolated colonies, no liquefaction
Lead acetate....			Negative for H_2S
Voges-Proskauer.....			Negative
Methyl red......			Negative
Gram............	Positive	Positive	Negative
Habitat.........	Stagnant water	Putrid and stagnant water	Muddy botton of brackish water
Optimum temperature......	20°C.	25°C.	18°C.

* According to Bergy's Manual.

virginianum 18°C. On the basis of these cultural differences this spirillum is described as a new species.

f. Discussion of morphology

Spirillum virginianum has a spiral, non-flexuous cell body consisting of ½ to 3 complete turns in young cultures, while in old cultures individuals are found which possess 7 turns. It is from 3 to 11 micra long and 0.6 to 0.9 micron wide. The spiral amplitude varies from 4 to 5.6 micra and the width of the spiral is 1.2 micron. The contours of the cell body are parallel and the ends are rounded, possessing a single, spiral flagellum at each end with the same spiral amplitude and width of spiral as the cell body. The spirilla appear barred when they are stained lightly, but solid when they are overstained.

Ehrenberg describes *Spirillum volutans* as possessing "a single whip-like flagellum" at each pole. Sternberg describes it as being from 1.5 to 2 micra wide and from 25 to 30 micra long; wave length from 9 to 13 micra; a single whip-like flagellum at each end, with opaque granules in the cytoplasm. Migula describes *Spirillum volutans* as a long, regular, spiral organism 30 by 1.5 micra, with spiral amplitude 6.6 to 7 micra and width of spiral 13 to 14 micra, 2 to 3½ turns, with 10 to 15 flagella at one pole. Hoelling is satisfied that it possesesses but one flagellum at each pole. Kutscher observed a tuft of flagella at each pole. Cohn reports only a single flagellum at each pole. Bergey describes *Spirillum volutans* as possessing spirals from 2 to 3 micra thick by 30 to 50 micra long, with 3 to 8 flagella at each pole. It is obvious that no two descriptions of *Spirillum volutans* are alike.

In 1909 Swellengrebel described in *Spirillum volutans* and in *Spirillum parvum* "finely honey-combed protoplasm with chromatin substance, distributed through the cytoplasm in transverse and zig zag bands." The description of these chromatin spirals stimulated much research and some controversy regarding the inner structure of the spirilla. Zettnow repeated Swellengrebel's work and confirmed the existence of "Chromatinbänder" in the cytoplasm of *Spirillum volutans*. Hoelling denied their existence, although his drawings certainly suggest it. He described the protoplasm as being alveolar. Swellengrebel's "finely honey-combed chromatin, distributed in zig zag bands," and Hoelling's "alveolar plasma" of *Spirillum volutans* appear to be the same structure, the apparent differences being due to dissimilar methods of staining.

In 1909 Fuhrmann described the flagellum in *Spirillum volutans* as "arising from the external membrane and meeting the cytoplasm in immediate union." In 1911–1912 Hoelling confirmed Fuhrmann's observations in every detail, but added "the flagellum penetrates the outer membrane by means of a hole." He further stated: "They must have their origin in the inner plasma and we differentiate them from the plasma. Therefore they consist of two elements, central elastic fiber, and an outer contractile plasma." Hoelling's final conclusion was that *Spirillum volutans* possesses only one flagellum at each pole.

A similar variation in the description of *Spirillum undula* is found in the literature. Sternberg describes it as a rigid organism having a spiral filament 8 to 12 micra long by 1.1 to 1.4 micron wide with spiral amplitude 4 to 5 micra, from ½ to 3 turns, and with a whip-like flagellum at each end. Migula describes it as a curved bacterium 4 to 5 micra long, ½ to 3 turns, and the number of flagella may reach 15. Bergey describes it as a stout, thread-like organism 1.2 to 1.5 micron wide by 8 micra long, spiral amplitude 4 to 5 micra, ½ to 3 turns. There are bundles of 3 to 9 flagella at each pole. It is obvious that these descriptions of *Spirillum undula* do not agree.

The morphological characteristics of these three types of spirilla are expressed in table 2, which shows two points of similarity between *Spirillum undula* and the spirillum described in this investigation. The first similarity is that they have the same wave length of the spirals, and the second is that they have the same number of turns. On the other hand, they differ in length and in the width of the cell bodies, in the number of flagella, and in the width of the spiral. Chromatin spirals are described in *Spirillum volutans* but not in *Spirillum undula.* The morphological characteristics presented in table 2 show that the organism in this paper is a new species. It is therefore named *Spirillum virginianum.*

Classification of Spirilla and Vibrios. It has been shown that the organism described in this investigation differs morphologically and physiologically from *Spirillum volutans* and *Spirillum undula.* On account of its rigid, spiral cell body, it can be classified under the genus *Spirillum.* On the other hand the presence of a single flagellum at each pole makes it possible to classify it under the genus *Vibrio.* Considering these two possibilities of classification this organism must be classified under the genus *Spirillum,* because it is curved in three planes, forms either long spirals or a portion of a turn, in contradistinction to the short, curved rods of the genus *Vibrio.* Furthermore, there is no valid reason why a spiral organism, possessing a single flagellum at each pole, should not be classified under the genus *Spirillum.* It is also clear from the original descriptions that the spirilla mentioned by Ehrenberg, Swellengrebel, Zettnow, Cohn, and Hoelling possessed a single flagellum at each pole, although the text books describe the spirilla as possessing tufts of flagella. Therefore this new organism is regarded as a spirillum.

TABLE 2

Comparative morphology of Spirillum volutans, Spirillum undula and Spirillum virginianum

	SPIRILLUM VOLUTANS (BERGY*)	SPIRILLUM UNDULA (BERGY*)	SPIRILLUM VIRGINIANUM
Length of cell	30–50μ	8.16μ	3–11μ
Width of cell	2–3μ	1.2–1.5μ	0.6–0.9μ
Spiral amplitude	6.6–7μ†	4–5μ	4–5.6μ
Width of spiral	13–14μ†		1–2μ
Number of turns	2½–3½†	½–3	½–3
Number of flagella	3–8	3–9	1
Chromatin spirals	Present		Present

* Description according to Bergy's Manual.

† Description according to Migula's Manual.

g. Discussion of coccoid bodies

The granules found in the cultures of spirilla are called "coccoid bodies" while similar structures found in cultures of spirochaetes are called granules. "Coccoid bodies" are common in old cultures of *Spirillum virginianum.* In hanging drop preparations they appear like single cocci, although larger than staphylococci. During certain stages of their life-cycle the "coccoid bodies" are motile and in their movements resemble very young tadpoles swimming about, although no flagellum could be seen in hanging drop preparations. Immotile "coccoid bodies" were also observed in hanging drop preparations made from old cultures of spirilla. "Coccoid bodies" with and without flagella were observed in stained preparations made from the same cultures. It is apparent that the motile "coccoid bodies" possess flagella, while the immotile do not. Plate 3 shows "coccoid bodies" in various stages of formation. Similar granules are described by Sorokin in the cell body of *Spirillum endoparagicum.* He interpreted these granules as analagous to spores.

In 1914 Wolbach and Binger described "bud-like forms either free or attached to the cell body" of the free living *Spirochaeta elusa.* They think that these buds are part of the life-cycle of this spirochaete.

In 1914 Todd and Wolbach studied the filterability of two strains of *Spirochaeta duttoni* and made some observations on the granules they found in their cultures. On the basis of their experiments these investigators came to the following conclusions: "The minute granules and comma bodies found in epithelial cells, and probably those in other tissues, are not stages in the development of spirochaetes. Large granules of coiled and encysted forms derived from spirochaetes, occur in various connective tissue structures, and may possibly represent resting or multiplication stages."

Meirowsky described red staining wart-like granules in the middle of *Spirochaeta nodosa* and in *Spirochaeta pallida.* He also reports granules in the living organisms and concludes that "they are the forms by which the spirochaetes sporulate in the process of multiplication." He further mentions bud-like granules attached to the cell body of the spirochaete. Finally he states that "the granules are part of the evolution cycle of the spirochaete."

Balfour has studied the granules in *Spirochaeta granulosa-penetrans* in connection with spirochaetosis of Sudanese fowl.

He first noticed granules in the erythrocytes of the peripheral blood of fowls suffering with spirochaetosis. Under the dark field illumination he observed spirochaetes entering the red blood cell and breaking into granules. He also described the pouring out of these granules by the host erythrocyte. His observations on the granules found in ticks infected with *Spirochaeta granulosa-penetrans* lead him to conclude that these granules develop into young spirochaetes. The granules that he observed in the blood of diseased fowl are described thus: "The spirochaetes shed the granules during a state of violent contortions, shaking themselves to and fro, like a dog shaking water from its coat." He succeeded in producing spirochaetosis in fowl by injecting them with mascerated ticks which showed only granules, and spirochaetes were found in the blood of the fowl in the course of the disease.

Dutton and Todd working with African Tick Fever were the first to describe coiled and encysted spirochaetes, containing a large number of small granules. Later Leishman advanced the view that the granules into which spirochaetes disintegrate are capable of developing into new spirochaetes. He laid particular stress on the significance of granules in the epithelial cells of the Malphighian tubules and in the ova. He further believes that the granules could multiply and were therefore not merely resting forms of spirochaetes, but represented a definite stage of the life history. Nuttall observed that *Spironema gallinarum* penetrates into the Malphighian tubules of infected ticks. There the spirochaetes break up into a large number of small coccoid bodies which he says multiply by fission. Hindle found that if infected ticks were kept at 21°C. no spirochaetes were present, but if kept at 35°C. they were. He further states that the granules develop into spirochaetes with a favorable temperature. On the other hand Wittrock and Couvy-Machoux brought much evidence to show that the granules above described were not connected with the spirochaetes. In the infected ticks the spirochaetes become very delicate and are not demonstrable with Giemsa stain, but they are by gentian violet, and by the dark field illumination.

CONCLUSIONS

1. The initial culture of these spirilla was obtained from the mud adhering to the outside of an oyster shell.
2. A medium consisting of 0.7 per cent beef extract agar containing egg cube medium was found satisfactory for growing them.
3. The spirilla were grown for many generations on 0.7 per cent beef extract agar before they were able to grow on the usual laboratory media.
4. The spirilla were isolated from the contaminating bacteria by plating them on 1.5 per cent beef extract agar containing egg cube medium and five days' incubation at 18°C. was necessary for producing colonies.
5. A combination of 2 cc. Sterling's anilin gentian violet, 2 cc. of 10 per cent basic carbol fuchsin in 60 cc. distilled water was a very satisfactory stain, while the flagella were stained well by first immersing the film in Fontana's solution no. 1.
6. The living spirilla possess a single, spiral flagellum at each pole; a refractive axial filament running longitudinally through the cytoplasm; and non-flexuous cell bodies.
7. Stained specimens show granular (barred) structure and chromatin spirals in the cytoplasm.
8. Motile and non-motile coccoid bodies were observed in hanging drop preparations from old cultures of spirilla.
9. Complete cycles in the process of transverse cell division were observed microscopically in hanging drop preparations.
10. The cultural characteristics were determined on the several laboratory media.
11. The name, *Spirillum virginianum*, is proposed as describing this new species.

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PLATES

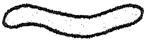
PLATE 1

FIGS. 1 TO 7. Illustrate the process of transverse division in *Spirillim virginianum* as observed in hanging drop preparation.

FIG. 3. Dotted lines indicate the bendings of the dividing spirillum.

FIGS. 4 AND 7. Dotted lines indicate the positions of the lashing daughter-cells in the process of division.

FIG. 8. A dividing spirillum stained by carbol fuchsin-gentian violet.



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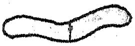

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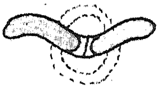

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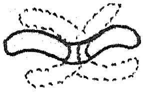

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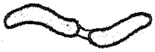

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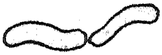

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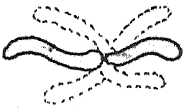

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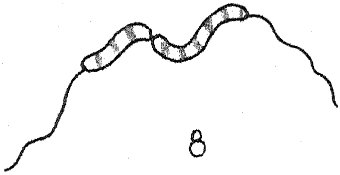

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(Dimitroff: *Spirillum virginianum* nov. spec.)

PLATE 2

Camera lucida drawings of *Spirillum virginianum* stained by carbol fuchsin-gentian violet.

FIGS. 1 TO 3. Branching (budding) forms of spirilla.

FIG. 2. Illustrates flagellum on each bud.

FIG. 4. Spirillum illustrating the formation of "coccoid body." Moist film.

FIGS. 5 AND 6. Degenerated specimens illustrating the cytoplasm segregated into the dark areas. The unstained areas are vacuoles.

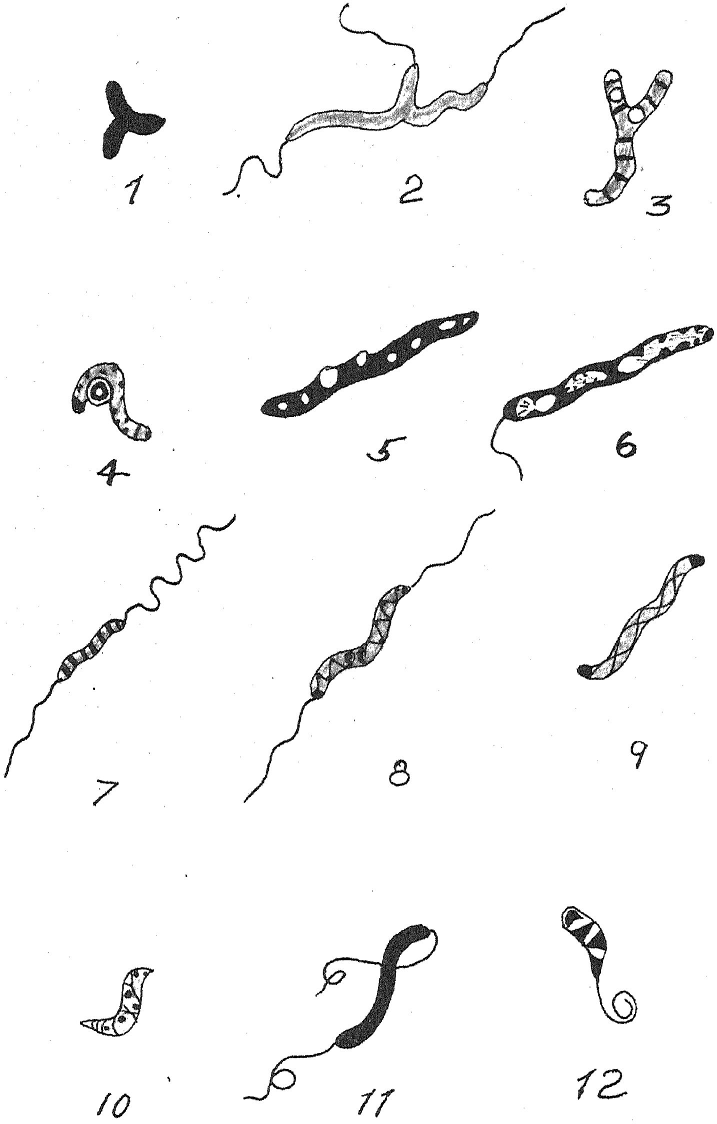
FIG. 7. Illustrates spirillum with transverse chromatin bars.

FIG. 8. Spirillum showing chromatin spirals and flagella. Stained intravitam.

FIG. 9. Spirillum showing chromatin spirals running in two directions, and polar kaps. Stained intravitam.

FIG. 10. Illustrates chromatin granules surrounded by chromatin septa. Stained intravitam.

FIGS. 11 AND 12. Spirilla with curled flagella. Air-dried films.



(Dimitroff: *Spirillum virginianum* nov. spec.)

PLATE 3

Camera lucida drawings of involution forms found in twenty-five-day pure culture of *Spirillum virginianum*. All the specimens were drawn from air-dried preparations stained by carbol fuchsin-gentian violet.

FIG. 1. Young individual possessing one flagellum.

FIG. 2. Involution form showing the contraction of the cytoplasm towards one pole.

FIG. 3. Illustrates the formation of "coccoid body" by the contraction of the cytoplasm towards one pole. The flagellum appears to arise from the cell membrane.

FIG. 4. Two "coccoid bodies" one at each end of what appears to be a flagellum.

FIG. 5. Spore-like spirillum with the cytoplasm at each pole.

FIG. 6. Degenerated, dead spirillum.

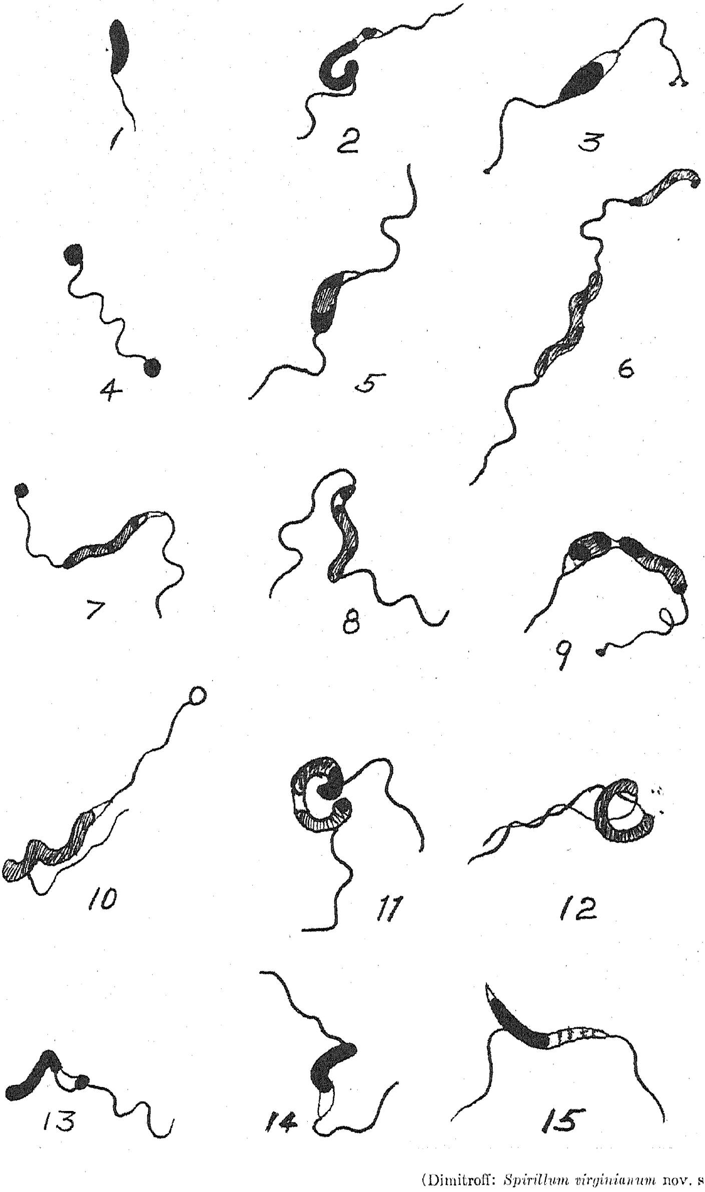
FIG. 7. Formation of "coccoid bodies" one at each end of the flagellum on the left as illustrated in figure 4.

FIG. 8. Spirillum showing the cytoplasm has segregated into small dots.

FIG. 9. Spirillum showing the cytoplasm has segregated into definite areas.

FIGS. 10 AND 12. Illustrate dead spirilla.

FIGS. 11, 13, 14 AND 15. Illustrate the contraction of the cytoplasm towards the poles.



(Dimitroff: *Spirillum virginianum* nov. spec.)

PLATE 4

Camera lucida drawings of *Spirillum virginianum* made from preparations stained by the intravitam method of moist films.

FIGS. 1 TO 7. The spirilla were stained with 1 per cent methyl green.

FIG. 1. Spirillum showing no chromatin.

FIGS. 2 AND 3. Spirilla illustrating the chromatin distributed in dots along the cell membrane of the spirillum.

FIGS. 4 AND 7. Illustrate chromatin spirals in spirilla as demonstrated by the intravitam method of staining.

FIGS. 5 AND 6. Illustrate the barred appearance of spirilla.

FIGS. 8, 10, 11 AND 12. Spirilla stained with 0.25 per cent malachite green.

FIGS. 8 AND 12. Illustrate chromatin spirals running in two directions. Polar kaps are shown.

FIG. 9. Illustrates chromatin spiral running in one direction.

FIG. 10. Shows the chromatin granules surrounded by chromatin septa.

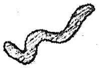
FIG. 11. Shows chromatin granules arranged along the cell wall, and a portion of the chromatin spiral is also shown.

FIGS. 13 AND 14. Spirilla stained with 1 per cent neutral red.

FIG. 13. Illustrates chromatin spirals.

FIG. 14. Shows chromatin spirals less prominent than in figure 13.

FIG. 15. Stained with 1 per cent safranin showing chromatin spirals and polar kaps.



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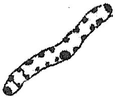

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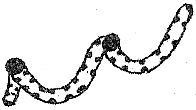

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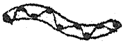

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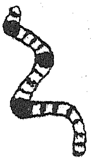

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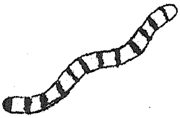

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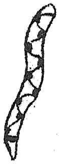

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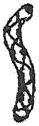

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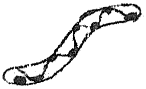

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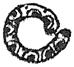

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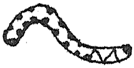

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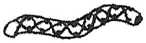

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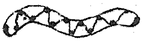

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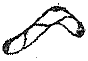

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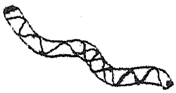

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(Dimitroff: *Spirillum virginianum* nov. spec.)

PLATE 5

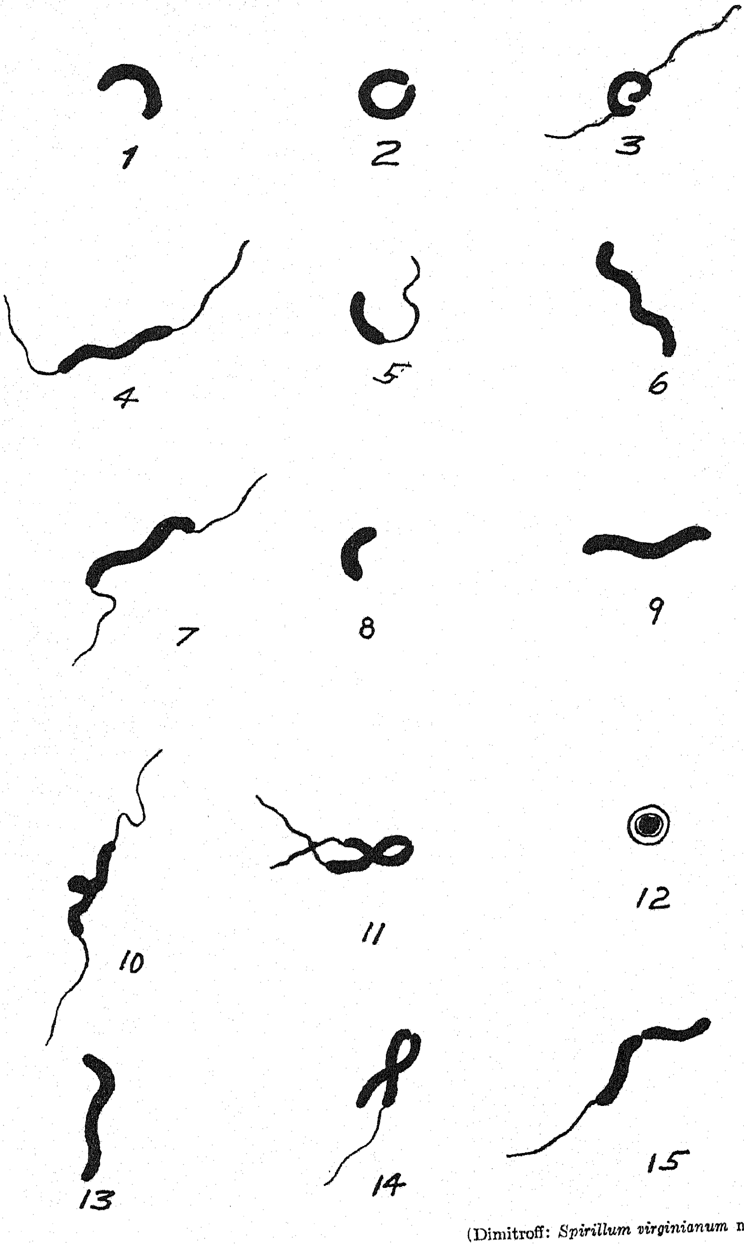
Camera lucida drawings of *Spirillum virginianum* made from air-dried films fixed by heat, immersed in Fontana's solution no. 1, stained in carbol fuchsin-gentian violet. This plate shows typical specimens of spirilla as found in young pure cultures of spirillum.

FIGS. 1 TO 3. Illustrate three stages in the formation of "coccoid bodies" by the coiling spirilla.

FIGS. 5 AND 8. Young spirilla.

FIGS. 4, 6, 7, 9, 10 AND 13. Normal, fully grown spirilla.

FIGS. 11, 14 AND 15. Dividing spirilla.



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THE ETIOLOGY OF A CANARY BIRD EPIZOOTIC[1]

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Organisms of the paratyphoid-enteritidis group are of wide distribution in nature, and it would seem that few well known mammalian species are free from invasion by them. Birds constitute no exception; witness, for example, *Bact. pullorum* infection in young chicks, *Bact. anatum* invasion of ducklings, and psittacosis of parrots.

The present work deals with an epidemic outbreak among canary birds kept in captivity, which occurred near New Brunswick during the month of April, 1924. The first appearance of this disease was in a bird store, where a stock of 200 birds became badly infected. The total loss in this store alone was about 70 birds, or a mortality of 35 per cent. Both young and old birds were affected. Another small breeder lost 25 birds out of a stock of 33. Several other birds in the city died during the same period. The source of the infection could not be determined, though it is possible that a lot of birds purchased shortly before the outbreak of the epidemic may have been responsible.

The first symptom of the disease was a "puffing up" of the birds. There were some indications of constipation and later a diarrhea developed, the droppings being of a greenish color. In some instances these droppings were decidedly bloody. In the last stages of the disease large amounts of urates appeared in the excreta.

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[1] Paper No. 256 of the Journal Series, New Jersey Agricultural Experiment Stations, Department of Poultry Husbandry.

Nine canaries which had died of the disease were brought to the laboratory of the senior author for examination. When received at the laboratory the specimens had decomposed somewhat, so that the color of the organs was altered. However, there was a catarrhal enteritis in all cases, with reddening of the mucosa. The contents of the intestines in some instances were bloody. The liver showed congestion, as did the lungs. The kidneys were also congested, and in some instances the ureters were distended. The spleen was not visibly affected. The heart muscle was dark red in color, but no hemorrhages could be found.

By means of a capillary pipette a small quantity of blood was removed from the heart and streaked on an agar plate. Cultures were taken from four of the birds on the day they were obtained. After twenty hours' incubation a pure growth was observed on each of the four plates. The following day the remaining five specimens were cultured, and in each case the heart's blood was found to contain the same organism, free from contaminating forms.

Transfers of the nine different strains were submitted to the junior author and a joint study of the organism was made.

THE ORGANISM

Stains made from agar slant cultures showed the organism to be a short Gram-negative rod. Neither spores nor capsules could be demonstrated. The growth on agar slant and agar plates was moderately abundant and resembled that of the colon-typhoid group. Gelatin was not liquefied by any of the strains after two weeks' incubation. An even clouding with slight sediment was produced in nutrient broth in twenty-four hours. Growth in litmus milk was accompanied by a slight transient acidity, the medium becoming alkaline in twenty-four to forty-eight hours. None of the strains produced indol in tryptophane broth. Nitrates were reduced to nitrites in twenty-four hours.

All of the nine strains of the organism were also examined for their ability to form acid and gas from carbohydrate media.

Acid and gas were produced in broth containing glucose, maltose, mannitol, levulose, arabinose, inositol, rhamnose, xylose, trehalose, galactose, and dulcitol. No acid or gas was produced in media containing lactose, sucrose, raffinose, inulin, salicin, adonitol, amygdalin and melezitose. All of the strains of the organism were apparently alike in their action upon carbohydrate media.

The fermentative properties of the organism link it closely with the *Bact. paratyphosum B.* group of bacilli. The fermentation of xylose and inositol would indicate that the organism was not of the *Bact. paratyphosum A* type. Jordan (1917) has pointed out that the majority of strains of *Bact. suipestifer* fail to ferment inositol, arabinose and dulcitol. It has been stated by Koser (1921) and Jordan (1923) that *Bact. suipestifer* does not ferment trehalose. The fermentation of trehalose, arabinose, inositol and dulcitol by the organism would therefore indicate that it is not *Bact. suipestifer*. Jordan has also stated that *Bact. enteritidis* fails to ferment inositol. If carbohydrate reactions may be taken as an index of the identity of an organism, the bacillus is evidently a member of the paratyphoid B. group.

TABLE 1

Serum of rabbit immunized against Bact. paratyphosum B (31)

ABSORBING ANTIGEN	AGGLUTINATES	1:100	1:400	1:1000	1:2000	1:4000
Unabsorbed.........	Canary I	++++	++++	++++	+	0
Unabsorbed.........	Para B (31)	++++	++++	++++	++++	++++
Canary I...........	Canary I	0	0	0	0	0
Canary I...........	Para B (31)	++++	++++	++++	++++	+++

TABLE 2

Serum of rabbit immunized against Bact. suipestifer (17)

ABSORBING ANTIGEN	AGGLUTINATES	1:100	1:400	1:1000	1:2000	1:4000
Unabsorbed......	Canary I	++++	++++	++++	++++	++
Unabsorbed......	Suipestifer (17)	++++	++++	++++	++++	++++
Canary I.........	Canary I	0	0	0	0	0
Canary I.........	Suipestifer (17)	++++	++++	++++	+++	+

AGGLUTINATION REACTIONS

The agglutinative relations of the canary organism were studied by the use of antisera prepared from known types of the paratyphoid group.

It was found that the serum of a rabbit immunized against *Bact. aertrycke* agglutinated the canary strains in the titer limit of the serum. This strain failed to agglutinate *Bact. enteritidis* but did cause a marked agglutination of *Bact. paratyphosum B.* and *Bact. suipestifer.* An immune *Bact. paratyphosum B.* serum caused a marked clumping of the canary strains as did an immune *Bact. suipestifer* serum.

It can be seen that the canary organism is agglutinated by sera prepared from *Bact. aertrycke, Bact. paratyphosum B.* and *Bact. suipestifer.* As is usually the case in the Salmonella group, the identity of the organism is masked by the large amount of cross agglutination.

TABLE 3

Serum of rabbit immunized against Bact. aertrycke (146)

ABSORBING ANTIGEN	AGGLUTINATES	1:100	1:400	1:1000	1:2000	1:4000
Unabsorbed......	Canary I	++++	++++	++++	++++	++++
Unabsorbed......	Aertrycke (146)	++++	++++	++++	++++	++++
Canary I.........	Canary I	0	0	0	0	0
Canary I.........	Aertrycke (146)	0	0	0	0	0

In order to determine definitely to which type the canary organism belongs, agglutinin absorption tests were carried out. The protocols (tables 1 and 2) show the results obtained when sera derived from *Bact. paratyphosum* B. and *Bact. suipestifer* were absorbed with the canary organism.

From the above protocols it can be seen that while the canary organism is agglutinated by sera derived from *Bact. paratyphosum B.* and *Bact. suipestifer*, it is not identical with either of these organisms. Absorption of these antisera with the canary organism left the agglutinins for the homologous bacilli almost unaffected.

However, when *Bact. aertrycke* antiserum was absorbed with the canary organism, different results were obtained, as is shown in table 3.

The absorption of *Bact. aertrycke* serum with the canary organism resulted in a complete removal of agglutinins for the homologous organism. The canary organism would seem, therefore, to be identical with *Bact. aertrycke.*

SOURCE OF CULTURES

Bact. aertrycke (146)—*Bact. pestis caviae* (146) Stock strain from Dr. Krumwiede. See Ferry, Jour. Pathol. and Bacteriol, 1924, **18**, 445.

Bact. aertrycke (MTII)—Mouse Typhoid II. See Amoss, Jour. Exper. Med., 1922, **36**, 25.

Bact. paratyphosum B (232}
Bact. paratyphosum B (234} Stock cultures from Dr. Krumwiede.

Bact. paratyphosum B (31)—Stock culture from Dr. W. G. Savage. Recovered from human paratyphoid fever.

Bact. suipestifer (17)—Stock culture from Dr. Theo. Smith.

Bact. suipestifer (290)—Stock strain from Dr. E. O. Jordan.

CONCLUSIONS

A cultural, morphological and serological study of a recent canary bird epizootic occurring among about 200 caged birds showed the etiological agent, a member of the paratyphoid-enteritidis group, to be identical with the type designated by some as *Bact. aertrycke* and by others as *Bact. pestis-caviae.*

The authors wish to express their grateful appreciation to Prof. L. F. Rettger for his helpful coöperation in this work.

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cellulose. The question of the decomposition of cellulose by bacteria has called forth considerable discussion, especially between Omelianski and Pringsheim, on the one hand, and Kellerman and his associates and Löhnis, on the other. Independent of this, a certain amount of definite information has accumulated concerning the nature of these organisms and the mechanism of decomposition of the complex polysaccharide. Only a few of these bacteria have been isolated in pure culture, but there is undisputed evidence that the organisms isolated are capable of breaking down celluloses. This process is greatly reduced in pure culture when compared with crude cultures. Thus far, little is known concerning the rôle that these organisms may play in the decomposition of celluloses in the soil itself, especially when compared with the fungi and actinomycetes.

HISTORICAL

No attempt will be made here to review in detail the subject in question; attention will be called only to some of the more important contributions. The first cellulose decomposing organisms to be considered among the bacteria were the anaerobic forms. By inoculating flasks, containing an inorganic solution and cellulose as the only source of energy, with river slime, Omelianski (1895–1897) demonstrated that cellulose "fermentation" took place in a short time, i.e., the cellulose was decomposed with the formation of acid and gas. The latter consisted of hydrogen, methane, and carbon dioxide. By the use of specific enrichment cultures, Omelianski (1904) succeeded in separating the hydrogen from the methane form, but every attempt to isolate the organisms in pure culture failed. A pure culture of an anaerobic bacillus capable of decomposing cellulose actively was finally isolated by Khouvine (1923) from human faeces. However, it still remains to be demonstrated whether the organisms of Omelianski and Khouvine play any rôle in the decomposition of cellulose in normal soils.

The ability of anaerobic bacteria to decompose cellulose was questioned by Kellerman and his associates (1912–1913), who isolated several aerobic bacteria capable of decomposing celluloses from the very cultures of Omelianski. The existence of various aerobic bacteria capable of decomposing celluloses in the soil was first demonstrated by Van Iterson (1904). This work was further extended by Kellerman and his associates (1913), McBeth and Scales (1913), McBeth (1916) and recently Sack (1924), who found that the soil harbors a number of bacteria capable of attacking cellulose aerobically. Omelianski (1913) and Pringsheim (1920) claimed, however, that Kellerman, McBeth and Scales have not established definitely that the aerobic bacteria which they have isolated are actually capable of decomposing cellulose. The fact that the only evidence submitted was the formation of a clear zone around the bacterial colonies on the cellulose plate and the maceration of paper in liquid media, and the fact that the transfer of the organisms upon starch and sugar media greatly reduced their cellulose decomposing power spoke in favor of Omelianski's criticism. Löhnis and Lochhead (1913) found that at least some of these, or similar, organisms actually broke down cellulose. The fact that the clear zone formed on the cellulose agar plate was actually a result of the formation of an exoenzyme, as claimed by Kellerman and his associates and Löhnis and Lochhead (1923), and not a result of the dissolution of the carbonate on the plate by an acid produced from the constituents of the tap water, as suggested by Omelianski, was recently confirmed by Bojanovsky (1925). However, the organism observed by this author (not isolated in pure culture) as well as the one studied by Merker (1913) and probably also that of Van Iterson (1904) strongly resemble, in description, the *Sp. cytophaga* H. and C., for which Hutchinson and Clayton (1919) have established undisputed proof of active cellulose decomposition. Waksman and Heukelekian (1924) demonstrated, by direct quantitative measurement of the amount of cellulose decomposed, that some of the bacteria isolated by Kellerman and his associates, like *Bact. fimi*, bring about a considerable reduction in the amount of cellulose in the soil or in artificial media under favorable cultural conditions. The nature of the cellulose decomposition by the *Vibrio agarliquefaciens* isolated by Gray and Chalmers (1924) and the bacteria of Sack (1924) still remains to be established.

The thermophilic bacteria capable of decomposing cellulose stand in a group by themselves. These organisms are characterized by their rapid action upon cellulose, as shown by Macfadyen and Blaxall (1899), Kroulik (1912), Pringsheim (1912–1913), and Fred and his associates (1924). In view of the fact that the process is at an optimum at 60°C., and since not many other organisms thrive at that temperature, the isolation of a pure culture can be readily effected.

The decomposition of cellulose by bacteria capable of reducing nitrates was first suggested by Van Iterson (1904), who believed that this is an anaerobic process. Groenewege (1920), however, demonstrated that this process, aerobic in nature, is carried out by the combined action of two organisms, one of which reduces the nitrate and the other decomposes the cellulose; the former utilizes the energy liberated by the latter which enables it to carry on the reduction process. The presence of the nitrate is not absolutely essential for the decomposition of cellulose, but other sources of nitrogen can also be utilized.

The earlier observations on the ability of fungi to decompose celluloses were made by plant pathologists, who investigated the penetration of the cell wall by various parasitic fungi. It is sufficient to mention the work of De Bary (1886), Marshall-Ward (1888), Kean (1890), Miyoshi (1894), Behrens (1898), Brown (1917) and Appel (1907), the latter having studied the decomposition of filter paper by Fusaria. Schellenberg (1908), however, doubted the validity of the claims of the earlier workers in regard to the decomposition of cellulose by fungi, since their studies were carried on on natural products in which hemicelluloses and not true cellulose were subject to decomposition. However, the work of Otto (1916), Mütterlein (1913), Kellerman (1913), McBeth and Scales (1913), Scales (1915), Daszewska (1924), Waksman (1916), Kosin (1921) and others established beyond doubt that fungi are capable of decomposing true celluloses. Some of these investigators (Mütterlein (1913), Scales (1915), Daszewska (1924)) even claimed that fungi play a leading rôle in the decomposition of celluloses in the soil.

Of course, not all the fungi are capable of decomposing celluloses. The various Mucorales, for example, including the genera Rhizopus, Mucor, Zygorhynchus and Cunninghamella, are unable to utilize celluloses as sources of energy (Waksman and Heukelekian, 1914). The fungi capable of attacking celluloses belong largely to the Fungi Imperfecti and Ascomycetes, and are found among the genera Fusarium, Aspergillus, Penicillium, Trichoderma, Verticillium, Cephalosporium and related forms, and certain Dematiaceae. There is also no doubt that various Basidiomycetes are capable of decomposing celluloses; it is sufficient to mention the organisms causing the decomposition of wood, etc.

The ability of actinomycetes to decompose celluloses was first established by Krainsky (1914). His results have been confirmed by McBeth and Scales (1913) and Waksman (1919). Conn (1916) observed a very extensive development of actinomycetes in sod soils, in comparison with cultivated soils, in old sod more than in new sod. He suggested, therefore, that these organisms play an important rôle in the decomposition of grass roots and other plant residues in the soil. Martin (1923) observed a similar increase in the numbers of actinomycetes as a result of the addition of various green manures (rye, oats, buckwheat) to the soil and he concluded that they are active in cellulose decomposition.

This brief review of the literature is sufficient to illustrate that the soil harbors a large number of organisms capable of decomposing celluloses; however, our knowledge is as yet insufficient to learn which of these organisms play the dominant rôle in the actual breaking down of celluloses in the soil, under different environmental conditions. The work of Scales (1915), Hill (1915), Bazarevski (1916), Murray (1921), and Waksman and Starkey (1924), taken together, tends to show that while the addition of straw, alfalfa meal, and other natural organic substances greatly stimulates the development of bacteria under aerobic conditions, the addition of cellulose to the soil does not markedly influence bacterial multiplication but brings about an extensive development of fungi, especially in soils acid in reaction. These results were obtained by the use of the plate method. Winogradsky (1924b) examined the soil flora by the direct microscopic method and found that the addition of cellulose to the soil greatly favored the development of fungus mycelium, while the addition of protein rich substances, glucose and mannitol favored the multiplication of bacteria,—in the last two instances of nitrogen-fixing organisms. On the other hand, there is no doubt that various bacteria are capable of decomposing celluloses. It remains to be found, therefore, under just what environmental and nutritive conditions this process is carried on by bacteria or by fungi, or whether the various groups of microorganisms may be active in the soil at the same time.

Very little information is available concerning the influence of reaction and soil moisture on the nature of the organisms capable of decomposing cellulose. Christensen (1915) found that the addition of $CaCO_3$ to acid peat soils was an essential requirement for cellulose decomposition; however, it is uncertain whether the effect was due to the direct influence upon the cellulose-decomposing organisms or upon the organisms liberating the nitrogen, an element essential for the growth of the former. Charpentier (1921) found that a difference in reaction produced by the addition of lime did not influence cellulose decomposition; however, the results of Waksman and Starkey (1924) seem to point to a decided difference in the development of bacteria on the one hand and fungi on the other in soils of different reaction: in the more acid soils the fungi predominated by far over the bacteria; in the neutral or alkaline soils, the proportional increase in the number of fungi was nearer that of bacteria. It is not known, however, whether the greater development of bacteria under neutral or alkaline conditions is due to a direct utilization of the celluloses or of the secondary products formed by the fungi or other organisms. Charpentier also found that cellulose decomposition was increased by an increase in the moisture content of the soil, but a further increase above the optimum brought about a reduction in cellulose decomposition. This optimum was near the point of saturation.

EXPERIMENTAL

Methods

The development of microörganisms in the soil can be followed either by the plate or by direct microscopic methods. In the case of bacteria, the plate method allows the determination only of organisms capable of development upon the particular medium used in the preparation of the plate. Obligate anaerobic cellulose decomposing organisms will not develop on the plate; it is also questionable whether all aerobic cellulose-decomposing bacteria will do so; certainly the active *Sp. cytophaga* does not. Unfortunately, however, our knowledge concerning the bacteria capable of decomposing cellulose in the soil is still so meagre that the methods available are insufficient even for the isolation and cultivation of these organisms, let alone their enumeration. One would, therefore, not expect that the bacteria developing as a result of addition of cellulose to the soil will be all found on the common plate used for counting bacteria in the soil. This is expecially true in the case of cellulose decomposition under anaerobic conditions, as shown later. The direct microscopic method, as developed by Conn (1918) and Winogradsky (1924a–1925), for counting the bacteria developing in the soil, still presents various difficulties. The egg albumen agar (Waksman, 1922) plate has been used in the following studies. The increase in the number of bacteria capable of developing on this medium, as a result of the addition of celluloses, does not necessarily indicate an increase in the number of organisms capable of utilizing celluloses directly as a source of energy, but may merely show an increase in certain heterotrophic soil bacteria, as a result of an increase of available nutrients, resulting either directly from the decomposition of celluloses or indirectly from the synthesizing activities of the cellulose-decomposing organisms. These nutrients allow a greater development of organisms capable of forming colonies on the egg-albumen medium. Were the results on the rôle of bacteria in the decomposition of cellulose in the soil based merely upon the plate method, they would be far from presenting a true picture of this process in the soil.

An increase in the development of fungi as a result of addition of cellulose to the soil can be determined both by the plate (Waksman, 1922b) and microscopic (Conn, 1922) methods. The fungi decomposing cellulose develop readily upon the plate and an increase in their number, as a result of addition of cellulose, indicates the direct utilization of cellulose as a source of energy by these organisms. However, the actual development of fungus mycelium in the soil does not necessarily go hand in hand with the increase in the number of fungi as determined by the plate method. A fungus colony on the plate is formed either from a piece of mycelium in the soil or from a spore. Those organisms that produce an extensive mycelium but few spores will give on the plate fewer colonies than those that produce a scant mycelium but a large number of spores. In this connection the direct staining method, suggested by Conn (1922), is of great help. When untreated soils are examined by this method, an occasional strand of fungus mycelium is found in a field, while a soil to which cellulose has been added is found permeated with hyphae. These are not found in long strands of mycelium, but in the form of short pieces of hyphae. This is due primarily to the fact that the Phycomycetes which form a long unicellular mycelium are unable to decompose celluloses and do not develop, therefore, to any extent as a result of addition of these materials to the soil. But the mycelium of the cellulose-decomposing fungi, including species of Fusarium, Trichoderma, Penicillium, and various Dematiaceae readily breaks up into short and more or less uniform pieces. A direct parallelism between the number of fungi as determined by the plate method and by the microscope was observed in the investigations reported here; this can, therefore, be readily explained.

For the determination of cellulose in the soil, the method of Charpentier (1921) as modified by Barthel and Bengtsson (1924) has been used.

Influence of available nitrogen upon cellulose decomposition in the soil

Niklewski (1912), Charpentier (1921), Barthel and Bengtsson (1924), and Waksman and Heukelekian (1924a) found that the decomposition of cellulose in the soil is largely controlled by the amount of available nitrogen. This is due to the fact that no nitrogen-fixing bacteria are capable of using cellulose directly as a source of energy; the organisms decomposing the cellulose, whether they be fungi or bacteria, need a definite amount of nitrogen for the synthesis of their protoplasm, using cellulose as a source of energy. The ratio between the cellulose decomposed and the nitrogen assimilated is about 30 to 1 in the case of fungi and probably also in the case of aerobic bacteria (Hutchinson and Clayton, 1919). The rate of nitrogen liberation in a soil will thus control the rate of cellulose decomposition. To hasten the latter process it is sufficient only to add available nitrogen in the form of nitrates or ammonium salts.

The above is well illustrated in the following experiment: One hundred gram samples of soil sieved through a 3 mm. sieve were air dried and placed in tumblers, 2 for each treatment. Water was added to bring the soil to 65 per cent saturation, also 1 gram of filter paper ground very fine into a powder, 200 mgm. $CaCO_3$, 50 mgm. K_2HPO_4, 20 mgm. $MgSO_4$, and different amounts of sodium nitrate. The moisture was kept constant by addition of distilled water every four or five days. After thirty days, the residual cellulose in the soil was determined (table 1).

These results confirm the observations made by Barthel and Bengtsson (1924) and Waksman and Heukelekian (1924a) that there is a direct parallelism between the available nitrogen added to the soil and the amount of cellulose decomposed. It also shows that nitrates offer a favorable source of nitrogen in this process. Barthel and Bengtsson (1924) found that the available nitrogen is alike for this purpose whether supplied in the form of stable manure or of ammonium sulfate. The nature of the nitrogen source is, therefore, not the controlling factor, while the amount of available nitrogen is. In the above experiment, 100 mgm. of sodium nitrate seemed to be just sufficient to bring about almost complete decomposition of cellulose. This experiment was repeated with another soil type with essentially the same results.

Influence of cellulose and straw upon the development of fungi and bacteria in the soil

Several experiments on the influence of moisture upon the decomposition of cellulose in the soil brought out the fact that the process was at an optimum at 40 to 100 per cent saturation; similar results were obtained by Charpentier (1921). In fully saturated soils, cellulose decomposition was very slow, especially at the early periods of incubation, but became active at a later period. This tends to indicate that the organisms capable of decomposing cellulose are found in normal aerated soils, where they find also optimum conditions for their activities, but they are not found abundantly or are comparatively inactive in soils saturated with water.

TABLE 1

*Influence of different amounts of sodium nitrate upon the decomposition of 1 per cent of cellulose in the soils**

$NaNO_3$ ADDED TO 100 GRAMS OF SOIL	SASSAFRAS SANDY SOIL		PENN LOAM	
	Residual cellulose in 100 grams of soil	Cellulose decomposed above check	Residual cellulose in 100 grams of soil	Cellulose decomposed above check
mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
0	776		806	
5	675	101		
10	644	132	691	115
25	468	308		
50	365	411	420	386
75	130	646	136	670
100	27	749	29	777
150	28	748		
200	42	734		
300	52	724		

* Period of incubation twenty-seven days.

In a preliminary experiment concerning the types of organisms which develop most abundantly as a result of addition of cellulose, a moist soil to which 1 per cent of ground filter paper and 0.1 per cent $NaNO_3$ has been added, was used. The results presented in table 2 indicate that the cellulose brings about a most extensive development of fungi, while it exerts hardly any effect upon the bacteria developing on the plate. This would tend to emphasize the rôle of fungi in the decomposition of cellulose in the soil. These results were confirmed very markedly by adding to pure sand 1 to 2 per cent straw or cellulose, the necessary minerals and nitrogen salt, inoculating with soil suspension and incubating; after three months incubation, the aerobic straw cultures had 156,000 fungi and 75,000,000 bacteria and the aerobic cellulose cultures 6,000,000 fungi and 2,600,000 bacteria. This is due to the fact that straw contains, in addition to celluloses, various substances (monosaccharides, starches, proteins) readily utilized by common soil bacteria, developing on the plate.

TABLE 2

Influence of 1 per cent cellulose upon the numbers of fungi and bacteria in the soil, as determined by the plate method

	BACTERIA		FUNGI	
INCUBATION OF SOIL + CELLULOSE, DAYS	15	30	15	30
Soil alone	12,640,000	13,500,000	3,110	4,150
Soil + 1 per cent cellulose	12,800,000	12,510,000	16,770	196,300

To throw further light on this subject, the following experiment was carried out using an acid forest soil: 1000 grams portions of air dry soil were placed in a series of glazed earthenware pots; 2 grams of $CaCO_3$ were added to each soil. Some of the soils were kept at 50 per cent water saturation and some at full saturation; some received 1 per cent cellulose in the form of ground filter paper or finely ground rye straw. Some received 0.1 per cent $NaNO_3$ and some did not. The pots were covered with glass plates and incubated at 25 to 28°C. At various intervals, samples were taken throughout the depth of the soil and analyses made of the residual cellulose and nitrate nitrogen, and of the numbers of bacteria and fungi. The results of the microscopic examination of fungi, using methylene blue, are represented as follows: 0, no hyphae found in any of several fields; 1, isolated strands of hyphae; 2, a few pieces in every field examined; 3 and 4, several or many pieces of hyphae in each field; 5, very abundant mycelium in all fields. These figures, given in table 3, represent only a series of gradations and not quantitative ratios.

The results indicate quite definitely that, under aerobic conditions, there is a distinct parallelism between the decomposition of cellulose and the development of fungi, also that both depend upon the presence of available nitrogen. In the absence of nitrate, the greatest development of fungi (twenty-six days) corresponded, under aerobic conditions, to the highest rate of cellulose decomposition. Similar results were obtained in the

TABLE 3

The influence of cellulose and straw upon the development of fungi in the soil, at different moisture contents, with and without $NaNO_3$

TREATMENT			FUNGI, THOUSANDS									
			Plate method					Microscopic method				
Nitrate added, 0.1 per cent	Organic matter added, 1 per cent	Moisture	3 days incubation	12 days incubation	26 days incubation	55 days incubation	85 days incubation	3 days incubation	12 days incubation	26 days incubation	55 days incubation	85 days incubation
		per cent										
0	0	50	57.5	55.6	54.4	59.0	49.0	1	1	1	1	1
0	0	100	45.3	20.0	18.0	7.0	3.0	1	1	?	0	0
+	0	50	64.0	69.0	62.0	59.0	68.0	1	1	1	1	1
+	0	100	40.0	22.0	24.7	5.3	3.2	1	1	?	0	0
0	Cellulose	50	73.0	88.5	120.0	63.5	62.0	2	3	3	3	2
0	Cellulose	100	49.5	35.0	20.0	7.4	4.2	2	1	?	0	0
+	Cellulose	50	106.0	340.0	340.0	346.0	240.0	2	5	4	3	2
+	Cellulose	100	40.0	23.0	20.0	4.2	3.1	1	1	?	0	0
0	Straw	50	177.0	356.5	440.0	495.0	350.0	3	3	3	2	2
0	Straw	100	31.7	15.3	9.5	17.5	4.5	2	3	1	0	0
+	Straw	50	332.0	910.0	1299.0	1490.0	990.0	4	5	4	4	2
+	Straw	100	13.0	19.0	4.0	3.9	1.5	2	1	?	0	0
Residual cellulose												
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*					
—	Cellulose	50	1.014	0.948	0.792	0.826	0.673					
—	Cellulose	100	0.955	0.804	0.669	0.429	0.176					
+	Cellulose	50		0.287	0.158	0.039	0.028					
+	Cellulose	100	0.949	0.953	0.794	0.544	0.217					

soils with straw, under aerobic conditions, except that bacterial development was much more abundant than in the case of cellulose. It is especially to be noted that a marked parallelism was found to exist between the numbers of fungi obtained by the plate method and the abundance of mycelium recorded by microscopic observations; the latter were always made while the plates were prepared, so that the numbers recorded could in no way influence the microscopic observations. The logical conclusion would, therefore, be that *the decomposition of celluloses and cellulose-rich substances in aerated soils is carried out largely by the agency of fungi; the fact that nitrogen becomes here a limiting factor would further indicate that nitrogen-fixing bacteria are unable to supply the nitrogen need in this process, by either utilizing directly or indirectly the energy made available in the decomposition of celluloses; also that the plate method can give a reliable index of the abundance of fungi in the soil.*

However, when the soil is saturated and covered with water, the numbers of fungi rapidly diminish, until they reach a minimum, independent of the addition of cellulose or straw to the soil, as demonstrated both by the plate and microscopic methods. The same is true of the actinomycetes. Neither of these groups of organisms plays, therefore, any rôle in the decomposition of celluloses and cellulose-rich substances in soils saturated with water or kept otherwise under anaerobic conditions. The decomposition of cellulose is here due entirely to the activities of bacteria. This cannot be demonstrated by the ordinary plate method, due to either of two reasons: 1. The bacteria decomposing the cellulose in soils saturated with water are anaerobic bacteria and, therefore, do not develop on the aerobic plate, or 2, these bacteria may be aerobic, but highly selective in their action and do not develop readily on the egg-albumen agar. The fact that abundant development of anaerobic bacteria takes place under these conditions can well be demonstrated by the use of the agar shake tube containing an appropriate medium. It is to be noted, however, that although the numbers of bacteria capable of growing on agar plates in the soils kept under anaerobic conditions constantly diminished (reduction in aerobic flora) after some time an increase began to take place in all cases (results not reported here). This is no doubt due to the adaptation of the proper organisms to the new environmental conditions. The most important phenomenon to be recorded in this connection is the fact that cellulose decomposition in the soil under anaerobic conditions (saturated soil) was more or less independent of the nitrate content of the soil. While under aerobic conditions, the amount of cellulose decomposed depended entirely on the amount of available nitrogen, as pointed out above and as illustrated in table 3, under anaerobic conditions, once cellulose decomposition sets in (it usually takes more time under anaerobic conditions before active decomposition sets in, due no doubt to the gradual adaptation of the proper bacteria), it goes on rapidly, independent of the quantity of nitrate nitrogen. This cannot be explained by the possible development of bacteria utilizing the by-products of cellulose decomposition and fixing atmospheric nitrogen, as one might be easily tempted to assume. It is explained entirely by the fact that under aerobic conditions cellulose is decomposed entirely to carbon dioxide and water thereby making available all its potential energy for the activities of microörganisms; under anaerobic conditions, however, cellulose is decomposed to various organic acids, largely butyric, and methane, CO_2 and hydrogen, as shown by Hoppe-Seyler (1889), Omeliansky (1904b) and in studies on the chemistry of cellulose decomposition under anaerobic conditions carried on in our own laboratory and which will be reported in a later paper; only a small amount of energy is thereby made available. Van Suchtelen (1923) calculated that, for 1 gram of CO_2 produced, 12 times as much heat is liberated in the complete oxidation of sugar as in butyric acid fermentation. We must also add that considerably less CO_2 is formed in the decomposition of cellulose under anaerobic than under aerobic conditions. Not only is the amount of energy liberated from the decomposition of cellulose under anaerobic conditions much smaller than in the decomposition of the same amount of cellulose under aerobic conditions, but the utilization of energy for the synthesis of protoplasm by the bacteria decomposing the cellulose under anaerobic conditions is also much less than in the case of fungi decomposing the cellulose under aerobic conditions. These two factors account for a much smaller amount of protoplasm synthesized and, therefore, of nitrogen required.

To demonstrate that anaerobic cellulose decomposing bacteria are not active under normal soil conditions, the following experiment was carried out. Sassafras soil, to which 1 per cent ground filter paper (weighed out into the individual flasks), calcium carbonate and traces of K_2HPO_4 and $MgSO_4$ were added, was placed, in 100 grams portions, into a series of Erlenmeyer flasks; the soil in some flasks was brought up with water to 60 per cent saturation and in others to 100 per cent. The flasks were plugged with cotton and sterilized, at 15 pounds pressure, for thirty minutes on two consecutive days. One hundred milligrams of sterile NH_4NO_3 was added to each flask. These were then inoculated with either 5 cc. of a suspension of soil, in which active cellulose decomposition was taking place, or with the same suspension which was heated for ten minutes at 80°C. This treatment killed the non-spore forming bacteria, fungi, and actinomycetes, but not the spores of the anaerobic bacteria. The results are given in table 4.

TABLE 4

Influence of heating soil suspension upon the decomposition of cellulose under aerobic and anaerobic conditions

INOCULATION	MOISTURE CONTENT	RESIDUAL CELLULOSE IN 100 GRAMS OF SOIL	CELLULOSE DECOMPOSED
	per cent	*mgm.*	*mgm.*
Uninoculated	60	1,020	0
	Saturated	1,042	0
Unheated suspension	60	27	993
	Saturated	583	459
Heated suspension	60	1,015	5
	Saturated	500	542

These results indicate that spore-forming bacteria are active largely under anaerobic conditions, but not under aerobic conditions. By examining the soils, in which cellulose had been decomposing, by the microscopic method developed by Conn and Winogradsky, no marked increase was found in the numbers of bacteria in the soil kept under aerobic conditions, while a marked increase was easily observed in the soils kept under anaerobic conditions. These results tend to confirm the idea previously expressed that, under anaerobic conditions, bacteria are largely responsible for the decomposition of cellulose in the soil.

Further studies on the nature of bacteria concerned in the decomposition of cellulose in the soil utilizing the silica gel plate method described in detail elsewhere (Waksman and Carey, 1926) revealed the fact that several groups of bacteria are capable of decomposing cellulose under aerobic conditions; these bacteria do not develop on the synthetic agar or nutrient agar plate and the use of the plate method is, therefore, valueless for demonstrating any direct increase in the number of cellulose decomposing bacteria in the soil. The most abundant and most common of these organisms was found to be the *Spirochaeta cytophaga*. These bacteria can be counted only by using a series of dilutions of soil, then adding 1 cc. portions of the various dilutions to cellulose-silica plates, incubating at 28° to 30° for five to seven days, then determining the abundance of bacterial development. By means of this method, large numbers of aerobic cellulose-decomposing bacteria are found in the soil to which cellulose-containing substances have been added.

The actinomycetes were not found to play any important rôle in the decomposition of cellulose in the soil. There was no marked increase in their development as a result of addition of cellulose to the soil. A number of cultures were isolated and tested; although some of them were found to be able to decompose cellulose, their action was very slow and could not compare with that of the fungi and bacteria. However, one phenomenon should be recorded in this connection. Although there was no increase in the number of actinomycetes during the period when cellulose decomposition was taking place, these organisms began to develop rapidly as soon as the cellulose was largely decomposed and the fungus mycelium began to disintegrate. This points to the rôle of these organisms as secondary agents of decomposition, attacking the bodies synthesized by the microörganisms, namely the fungi and bacteria which decomposed the celluloses as well as intermediary products of decomposition. This would tend to throw further light upon the succession in the soil population and upon the rôle played by the different members of the population in the various soil processes.

TABLE 5

Influence of partial sterilization of soil upon the members of fungi and bacteria and upon the decomposition of cellulose

CELLULOSE, 1 PER CENT	$NaNO_3$, 0.1 PER CENT	CS_2 TREATMENT	REINOCULATED WITH SOIL	INCUBATION	FUNGI PER GRAM SOIL	BACTERIA PER GRAM SOIL	CELLULOSE DECOMPOSED
				days			*per cent*
−	−	−	−	15	77,000	13,300,000	−
−	−	−	−	30	83,000	13,300,000	−
−	+	−	−	15	80,000	13,600,000	−
−	+	−	−	30	73,000	12,500,000	−
−	−	+	−	15	0	19,200,000	−
−	−	+	−	30	0	40,800,000	−
−	−	+	+	15	224,000	241,300,000	−
−	−	+	+	30	724,000	314,000,000	−
+	−	−	−	15	99,000	14,600,000	39.0
+	−	−	−	30	178,000	17,800,000	51.0
+	−	+	−	15	0	20,400,000	6.5
+	−	+	−	30	0	37,400,000	0
+	−	+	+	15	800,000	183,000,000	68.0
+	−	+	+	30	1,040,000	262,000,000	84.5
+	+	−	−	15	330,000	16,700,000	71.0
+	+	−	−	30	590,000	15,800,000	92.0
+	+	+	−	15	0	28,300,000	0
+	+	+	−	30	0	47,600,000	0
+	+	+	+	15	620,000	152,000,000	83.0
+	+	+	+	30	2,120,000	252,000,000	92.5

The influence of elimination of fungi on the decomposition of cellulose in the soil

To obtain further light on the rôle of fungi in the decomposition of cellulose in the soil, an attempt was made to study this process in a soil from which the fungi were eliminated. This can be done by treatment of the soil with volatile antiseptics, as shown, among others, by Waksman and Starkey (1923). The spore-forming and non-spore-forming bacteria are at first repressed and later develop in numbers greatly above those present in the untreated soil. It takes a considerable period of time before the fungi become again reëstablished.

A number of 100 gram portions of a calcareous air dry soil were placed in 250 cc. Erlenmeyer flasks. One per cent portions of ground filter paper and 100 mgm. portions of $NaNO_3$ were added to some of the soils. Some of the flasks with and without cellulose were treated with 5 cc. of CS_2 for seventy-two hours; the stoppers with which the flasks were closed during the treatment were then replaced with cotton plugs and the CS_2 allowed to evaporate. Sterile distilled water was added when necessary. Some of the flasks were reinoculated twenty-four hours after the treatment with 1 cc. soil suspension. The results are given in table 5.

The results show quite distinctly that strong cellulose decomposition goes hand in hand with the development of fungi. The soil partially sterilized and reinoculated was especially vigorous in the development of fungi active in the decomposition of cellulose. The bacterial numbers did not vary greatly, except that they were stimulated by partial sterilization. A direct examination of the soil for fungus hyphae gave results very much similar to those obtained by the plate counts. When the fungi are eliminated from the soil, even though bacterial development is greatly stimulated, cellulose decomposition is brought to a standstill. Extensive development of fungi and decomposition of cellulose took place in the partially sterilized and reinoculated soils even when no available source of nitrogen was added; this is due to the fact that the treatment of the soil with a volatile antiseptic results in an increase in the store of available nitrogen, as shown by the numerous workers on partial sterilization of soil. These results do not tend to confirm the idea advanced by Russell and Hutchinson (1911–1913) that the favorable effects of partial sterilization of soil are due to the elimination of protozoa; they rather tend to throw further light upon the rôle of fungi in the soil and the nitrogen stored in their mycelium as well as in the tissues of the worms, protozoa, insects, etc., inhabiting the soil which are killed by partial sterilization, an idea advanced by Störmer (1907), Stoklasa (1911), Waksman and Starkey (1923) and others. The fact should not be overlooked, however, that certain cellulose-decomposing bacteria, like *Sp. cytophaga,* are also injured by treatment with volatile antiseptics.

To demonstrate whether the fungi, which form colonies on the plates prepared from soil in which active cellulose decomposition has taken place under aerobic conditions, are capable of decomposing cellulose in pure culture, a number of these organisms were isolated from the plate and cultivated in pure culture. The pure cultures were tested for their cellulose decomposing power by inoculation into sterilized soil, to which 1 per cent cellulose and 0.1 per cent NH_4NO_3 had been previously added; in all 16 different organisms were tested and were all found capable of decomposing the cellulose, leaving only 2 to 10 per cent of the cellulose undecomposed, after an incubation period of three to four weeks. A series of bacteria isolated from a plate, prepared from a soil in which active cellulose decomposition has taken place, including gram-positive and gram-negative rods and cocci and spore forming bacteria, were also tested by the same method and were found to be unable to decompose cellulose.

This tends to confirm, of course, the previous observations that the bacteria developing on the plate have nothing to do directly with cellulose decomposition and are active, as far as transformation of organic matter in the soil is concerned, in secondary processes. When crude organic materials are added to the soil, these bacteria attack the monosaccharides, starches, proteins and other available substances. The true cellulose bacteria do not develop on the plate and any attempt to use the common plate method for determining the influence of celluloses upon the development of specific bacteria in the soil is useless. The development of fungi can, however, as we have seen, be detected very readily by the plate method.

Further information in regard to the organisms concerned in the decomposition of cellulose and straw can be obtained by the hanging drop method. A drop of a synthetic nutrient solution is placed upon a cover slip and a small piece of filter paper or straw is added to the drop. The preparation is then inverted over a cavity in a slide, into which a drop of water has been introduced; a little vaselin may be used; a slight opening should be left to insure aerobiosis. The drop of liquid upon the cover slip may be inoculated with a sterile needle from a dilute soil suspension. The preparation is examined under the microscope at frequent intervals. The piece of paper is rapidly attacked by fungi in the presence of nitrogen. In the absence of nitrogen, it remains practically sterile: no bacteria are found to attack the paper until some considerable time has elapsed. They appear even then to be attacking not so much the paper or any intermediary products, but the fungus mycelium. The hyphae of the fungi disappear after some time and their place may be taken by a strand consisting of bacterial cells. In the case of straw, however, the bacteria are the first to come in and are soon followed or accompanied by the fungi. Bacterial development is then very abundant, even if no additional nitrogen has been added.

DISCUSSION

The results presented in this paper, taken together with those obtained by other investigators, point definitely to the important rôle played by fungi in the decomposition of cellulose in normal soils. In arid and in alkaline soils, the aerobic bacteria are probably very important, while in soils saturated with water, the anaerobic bacteria play the predominant rôle in the decomposition of the cellulose. In normal soils, the anaerobic bacteria do not exert any influence on the decomposition of celluloses. Actinomycetes are also capable of decomposing cellulose, but their action is slow and in aerated acid soils they are rapidly crowded out and overgrown by the fungi. Thermophilic bacteria are also capable of decomposing celluloses rapidly, but their rôle is probably largely limited to the decomposition of straw in the manure pile rather than in normal soils.

The question of decomposition of straw which contains, in addition to cellulose, also monosaccharides, starches, proteins, pentosans, lignins, fats, and waxes, tannins, and other substances will be taken up in a later paper; it is sufficient to state here that the different ingredients of the straw are rapidly attacked by various bacteria, while the fungi attack these and also the celluloses. This is the reason why Krüger and Schneidewind (1899–1900) and Hiltner (1908) found that partial sterilization of soil was sufficient to prevent the injurious effect of straw upon crops. This injury is due entirely to the utilization of the straw as a source of available energy; the bacteria and fungi rapidly utilize this energy and large quantities of microbial protoplasm are synthesized. In view of the fact that 20 to 40 per cent of the carbon of the cellulose may be used by the microörganisms for the synthesis of their protoplasm and, since this protoplasm may contain 3 to 10 per cent nitrogen, large quantities of this element in an available form will be required. According to Waksman and Heukelekian (1924), 1 unit of nitrogen will be required by fungi (also by bacteria) for every 30 units of cellulose decomposed. If the same holds true of straw and if the celluloses, pentosans and other carbohydrates of the straw are as readily available sources of energy as ground filter paper is, 100 units of straw will contain a minimum of 60 units of available carbohydrate (leaving out the fats, waxes, and lignins); the complete decomposition of this material by fungi and bacteria will require a minimum of 2 units of nitrogen; straw in itself contains only 0.3 to 0.5 per cent nitrogen. At least three to four times as much nitrogen will have to be obtained by the microörganisms in the process of decomposition of the straw from outside sources; this will produce a nitrogen starvation for the growing plant. The greater the amount of straw added to the soil and the less the amount of available nitrogen which the soil is capable of liberating in a given period of time and under a given set of conditions, the sooner will the phenomenon of nitrogenous starvation set in. The addition of available nitrogen in the form of nitrates and ammonium salts will remedy this defect, wholly or in part, as shown by Krüger and Schneiderwind (1899–1900), Bischoff (1914), Sievers (1921), Söderbaum and Barthel (1924).

The idea advanced by Pringsheim (1910), Koch (1910), McBeth (1913), Dvorak (1912), Hutchinson and Clayton (1919) and others, that celluloses are used as sources of energy by nitrogen-fixing bacteria, in the presence of cellulose-decomposing forms, is not borne out by these studies, when the fungi are the forms largely concerned in the decomposition of cellulose. Were these organisms capable of supplying energy to nitrogen-fixing bacteria, there would be no nitrogen starvation, as a result of addition of cellulose-rich substances to the soil; the fact that nitrogen starvation does set in and the fact that there is no increase in the nitrogen content of the soil as a result of the addition of these substances speaks against nitrogen fixation under normal aerobic soil conditions, with cellulose as the only source of nitrogen. In the presence of nitrogen, the fungi decompose the cellulose completely (Heukelekian and Waksman, 1925); in the absence of nitrogen, they do not attack it at all. In the case of straw, however, some of the soluble carbohydrates may be used as sources of energy for nitrogen-fixing bacteria. There is some evidence, both in the literature and in our own investigations, that when large amounts of straw and especially substances rich in lower carbohydrates, polyhydric alcohols and starches and poor in nitrogen are added to the soil, the immediate reduction in the available nitrogen of the soil will stimulate the activities of the nitrogen-fixing bacteria, such as *Azotobacter* and *Bac. amylobacter*, and the amount of nitrogen fixed will be in direct proportion to the amount of carbohydrate directly available as a source of energy to these organisms. Cellulose is decomposed in the soil only when available nitrogen is present and the amount of decomposition is limited by the amount of this available nitrogen. The latter is assimilated by the fungi and bacteria, and transformed into microbial proteins. It is possible that when bacteria are the active agents in cellulose decomposition, certain intermediary and synthesized products may be utilized by nitrogen-fixing bacteria as sources of energy.

Large quantities of nitrogen are thus stored away in the bodies of the microörganisms decomposing the celluloses and other carbohydrates added to the soil. This nitrogen is readily made available as soon as the organisms die and are in their turn decomposed, as shown by Starkey (1924). Studies on the chemistry of cellulose decomposition under aerobic and anaerobic conditions and on the bacteria concerned in this process under anaerobic conditions will be published in detail later.

SUMMARY

1. Fungi, aerobic bacteria and anaerobic bacteria are the most important agents concerned in the decomposition of celluloses in the soil.

2. The amount of cellulose which is decomposed in the soil under aerobic conditions is dependent upon the amount of available nitrogen; under anaerobic conditions, a much smaller amount of nitrogen is required for the decomposition of the same amount of cellulose than under aerobic conditions; this is due to the much smaller amount of energy liberated and utilized by the organisms decomposing cellulose under anaerobic conditions. The optimum moisture concentration for the aerobic decomposition of cellulose is from two-thirds saturation to saturation.

3. Aerobic decomposition of cellulose sets in very early if available nitrogen is present; anaerobic decomposition of cellulose sets in late, after a continued lag period, indicating that in normal soils, organisms capable of decomposing cellulose under anaerobic conditions are not found in great abundance.

4. The addition of cellulose to soils kept under aerobic conditions, especially in the presence of available nitrogen, causes a very large increase in the number and development of filamentous fungi, as determined by the plate count and direct microscopic examination.

5. There is a direct correlation between cellulose decomposition, development of fungi and transformation of soluble nitrogen into microbial protoplasm.

6. Most of the bacteria developing on the plate obtained from a soil in which cellulose decomposition has taken place, are unable to decompose cellulose in pure culture. By special methods, such as the silica plate method, aerobic bacteria capable of decomposing cellulose are easily demonstrated in great abundance.

7. The elimination of fungi from the soil by treatment with volatile antiseptics prevents cellulose decomposition in the particular soil, under aerobic conditions; reinoculation of the partially sterilized soil brings about an extensive development of fungi and a rapid cellulose decomposition.

8. Under anaerobic conditions, both in the soil, in the straw heap and in the manure pile, bacteria are entirely concerned in the decomposition of cellulose.

9. Aerobic bacteria are capable of decomposing cellulose in the soil and probably play an important rôle in this process in certain soils.

10. Some actinomycetes are capable of decomposing celluloses but they do not seem to play any direct part in this process in the soil itself; their action is largely limited to the secondary products, either liberated or synthesized, and to some constituents of natural organic substances.

11. The fact that nitrogen becomes the limiting factor in the decomposition of cellulose in the soil shows that nitrogen-fixing bacteria probably do not increase the store of soil nitrogen, under aerobic conditions, especially in humid soils, when celluloses and straw are added as sources of energy. Nitrogen fixation takes place, however, when starches and lower carbohydrates, which are available sources are also introduced.

12. These results lead us to conclude that, under aerobic conditions and in humid soils, fungi are largely concerned in the decomposition of celluloses. In arid and alkaline soils, aerobic bacteria seem to play an important part in the decomposition of celluloses. Under anaerobic conditions, bacteria are solely concerned in the decomposition of celluloses. This is true not only of soils, but probably also of the decomposition of celluloses under all natural processes.

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NOTES AND QUERIES

As the result of a demand from various members of the Society it has been decided to initiate a column of Notes and Queries in the JOURNAL. If the response of members and subscribers seems to warrant it one or two pages of each issue of the JOURNAL will be set aside for brief questions, answers and communications bearing on bacteriological problems, each such question, answer or statement to be limited to a maximum of 50 words, the editor reserving the right to reject or condense any material which may be submitted. Material for this column should be sent to C.-E. A. Winslow, Yale School of Medicine, New Haven, Conn., and will be given publication as promptly as circumstances permit. In answering queries please note the number of the query and the issue of the JOURNAL to which the answer applies. If replies are made directly to the author of a question, it is requested that a copy of the reply be sent to the editor of the JOURNAL.

1. A correspondent desires to know if there is conclusive evidence in existence as to whether mitochondria are or are not of bacterial nature, and would appreciate references to sources of information on this subject.

2. Mr. Paul L. Schwartz, 95 A Street, Edmonton, Alberta, Canada, desires references to any work done on *Saccharomyces guttulata* other than that reported by Guilliermond and Lafar.

Professor Jean Broadhurst of Teachers College, Columbia University, New York City, desires answers to the six questions below:

3. Among all the so-called red yeasts in the various collections throughout the United States is there one in which the pigment is really blood red and not salmon pink?

4. Is there any in which the pigment is really water soluble?

5. Are there any methods now in use for reducing without wholly preventing evaporation of water through porous earthenware petri dish tops (other than glazed tops).

6. Are there individuals or laboratories now in possession of bacteria with water soluble pigment, other than *B. pyocyaneus*.

7. Who has chlorine-resistant organisms, which survive swimming pool treatment consistently?

8. What non-spore forming organisms are found habituating themselves to mechanical or rapid sand filters?

THE USE OF THE SILICA GEL PLATE FOR DEMONSTRATING THE OCCURRENCE AND ABUNDANCE OF CELLULOSE-DECOMPOSING BACTERIA[1]

SELMAN A. WAKSMAN AND CORNELIA CAREY

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The question of the identity of the organisms concerned in the decomposition of cellulose in nature has long attracted the attention of the microbiologist, due to the great predominance of this group of polysaccharides in nature and to the important rôle that microörganisms play in its transformation into simpler compounds. The ability of fungi to decompose celluloses and the part played by actinomycetes has been pointed out elsewhere (Waksman and Skinner, 1926). It is the question of cellulose decomposition by bacteria which has been the subject of most active controversy; and, even at present, while the bacteria capable of breaking down celluloses are classified into at least five groups (Pringsheim, 1923), a great deal of confusion still exists concerning their identity and interrelationship.

Without going into a detailed review of the literature, attention may only be called to the following facts: 1. There is no definite proof that anaerobic bacteria are capable of decomposing cellulose, at least in normal soils. Cellulose can no doubt be decomposed very actively under anaerobic conditions, but it still remains to be proven whether this process is carried out by purely anaerobic forms or by an association of aerobic cellulose-decomposing bacteria and anaerobic forms which utilize the products of the former. Khouvine (1923) is the only worker who has undoubtedly succeeded in isolating an anaerobic organism, *Bact. cellulosae-dissolvens*, capable of decomposing cellulose actively (much less vigorously, however, in pure than in crude culture).

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[1] Paper No. 258 of the Journal Series, New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

This organism was isolated from the human intestine and grew only in the presence of fecal extract; it still remains to be proven to what extent it is capable of breaking down celluloses in nature, as in soil, manure, sewage purification, etc. 2. There is no doubt that a large number of aerobic bacteria, including spore-forming and non-spore-forming organisms, rods and cocci, are capable of attacking cellulose, but experimental proof concerning the rapidity of their action and the biochemical processes involved is still insufficient. This is largely due to the slow growth of these organisms and the comparatively slow decomposition of cellulose upon the agar plate (McBeth, 1916); the presence of zone formation and even of maceration of cellulose in liquid media is hardly a sufficient index of these processes as they may take place in nature. 3. Although there is no doubt that certain thermophilic bacteria are capable of decomposing celluloses and although these organisms are abundantly distributed in nature, it is doubtful whether, outside of composting of manures, they are active in normal natural processes, such as in the soil. 4. The same is true of cellulose decomposition in association with nitrate reduction. 5. The isolation of *Sp. cytophaga* by Hutchinson and Clayton (1919), an organism capable of decomposing celluloses using various inorganic salts as sources of nitrogen, was of great interest, not only on account of the specific morphology and physiology of the organism, which place it in a class by itself, but largely because of the fact that this organism is aerobic and decomposes cellulose at 30°C. with great rapidity, forming a mucilage and organic acids, but no gas; the products can be utilized as sources of energy by nitrogen-fixing bacteria.

The greatest difficulty experienced in the isolation of cellulose-decomposing bacteria has been the lack of an appropriate simple medium, which would allow a rapid development of the organism and which could also be used for the study of its morphological and physiological activities. Such a medium seemed to have been offered by the cellulose agar of Kellerman and associates (1913 to 14) and McBeth (1916). However, this medium is rather tedious to prepare; the use of agar will allow the development also of other organisms which have nothing to do with cellulose decomposition. The fact that the bacteria isolated from cellulose plates and cultivated in common media were greatly reduced in their cellulose-decomposing power led Omelianski (1913) to suggest that the true cellulose bacteria were never isolated by Kellerman and McBeth, but that they were studying only contaminating forms. This is further emphasized by the fact that one of the few bacteria isolated in pure culture, whose identity cannot be doubted, namely the *Sp. cytophaga* H and C, does not grow on media not containing cellulose and is even injured by certain concentrations of sugars and protein degradation products. Löhnis and Lochhead (1923) found that *Sp. cytophaga* plays a predominant rôle in cellulose decomposition, under aerobic conditions, especially when the numerous other aerobic bacteria of a lower cellulose-decomposing efficiency are eliminated, by long continued cultivation and purification of the mixed growth obtained in the enrichment experiments.

These considerations suggest that only specific and selective media are desirable for the demonstration, isolation, and study of bacteria decomposing celluloses. Silica gel media which are free from any traces of impurities and contain only the specific nutrients could, therefore, prove ideal for this purpose. This was the medium which was utilized by Winogradsky in his brilliant contributions on the isolation of nitrite and nitrate forming bacteria. Winogradsky (1925) has demonstrated recently that silica gel plates containing the specific nutrients can also be used for the direct isolation from the soil of Azotobacter, "humivorous" and other specific soil bacteria.

While the following investigations were in progress, a paper appeared by Bojanovsky (1925) in which he used silica gel media for the isolation of cellulose-decomposing bacteria. An aerobic organism was isolated upon this medium consisting of two forms, a rod and coccus; since these two forms could not be separated from one another, the author suggested that he had here a symbiotic relationship of two organisms, which participate both in the decomposition of cellulose. This, as well as the nature of growth and the results of our own investigations, suggest that he was probably dealing with the *Sp. cytophaga.*

EXPERIMENTAL

Anyone who has had experience with the preparation of silica gel will appreciate the difficulties encountered in this connection. Of the various dialyzed, semi-dialyzed and undialyzed preparations, the original one of Winogradsky (1925) can still claim first place in simplicity and ease of preparation. It consists in pouring a solution of potassium silicate, sp. gr. 1.06 (B 6 to 8), into an equal volume of hydrochloric acid, sp. gr. 1.10 (B. 13), mixing well, distributing into dishes and allowing the gel to solidify; then placing the dishes in running water, until the wash water is free from chlorides and is neutral to brom-cresol purple. The dishes are then washed several times with boiled distilled water. This method has been somewhat modified, changing the concentration of silicate and acid so as to obtain a solidified gel within a few minutes rather than days as in the method of Winogradsky. After several modifications, the following method was found to give the best results:

A normal solution of hydrochloric acid and an equivalent solution of C. P. potassium silicate solution (about 10 per cent silicate) are prepared. Several 5 cc. portions of the hydrochloric acid are then placed into a series of test-tubes and varying amounts of the silicate solution ($4\frac{1}{2}$, $4\frac{3}{4}$, 5, $5\frac{1}{4}$, $5\frac{1}{2}$, 6 cc.) are added to the numbered tubes. The contents of the tubes are immediately mixed and poured into a series of dishes. The dish where gel formation takes place within two to five minutes is selected. The silicate solution is then diluted with distilled water so that equal volumes of the silicate and acid give a gel in the desired period of time. To prevent solidification during the process of gel formation, small quantities are mixed at a time. One hundred to 200 cc. of the silicate solution is poured into an equal volume of the acid solution and well mixed. The mixture is then poured into the lower halves of deep Petri dishes of ordinary bacteriological size; enough of the mixture is added to form a layer of about 3 to 4 mm. (requiring about 30 cc. per dish). The dishes are allowed to rest on a level surface until the gel is well formed. They are then placed in deep flat vessels and dialyzed in running tap water until free from chlorides. About twenty-four hours is required for this purpose. The dishes are then removed and transferred to a sterile vessel containing boiled distilled water. This is replaced several times. After they have been properly washed, the dishes are drained and treated with the nutrient medium. The cellulose used for the preparation of the plate consists of thoroughly ground filter paper, suspended in a medium of the following composition:

$(NH_4)_2HPO_4$	5.0 gram
$MgSO_4$	1.0 gram
$FeSO_4$	0.02 gram
KCl	1.0 gram
Distilled water	100 cc.

Five grams of the paper suspended in 100 cc. of this medium will suffice for the preparation of some 50 plates. About 2 cc. of the suspension of cellulose in the medium is poured upon the surface of each plate, in such a manner as to have the cellulose uniformly distributed over the surface; some $CaCO_3$ is then powdered on over the whole surface of each plate. The uncovered plates are placed in a thermostat at 65°C., until the excess of water has evaporated, without allowing the plates to become dry. The dishes are covered with sterile tops and are kept until needed for use.

A good growth of the bacteria capable of decomposing cellulose can best be obtained by inoculating particles of soil directly upon the properly prepared plates. This method has been suggested by Winogradsky (1925) for his auxiliary cultures. Crude cultures may also be obtained by inoculating liquid cultures, containing paper as the only source of energy, with soil. By using the first method, small particles of soil are pressed into the gel, in several places over the plate. Ordinary fertile field soil or greenhouse soils can be used; a soil, in which 1 per cent of ground filter paper and 0.1 per cent ammonium phosphate or nitrate have been incorporated a month previously and kept at optimum moisture and temperature, is, however, best suited for this purpose. The presence of cellulose decomposing bacteria will be manifested within forty-eight to seventy-two hours, by the appearance of a yellow or orange colored growth around the soil particles rapidly spreading over the plate. When examined under the microscope, the growth is found to consist of various bacteria and protozoa, later also accompanied by actinomycetes. When the growth has proceeded for four to five days, transfer is made from the edge of the growth into flasks containing about 1 gm. of filter paper and 25 cc. of medium of the above composition (without $CaCO_3$), the flasks being previously sterilized. Various dilutions of the culture can thereby be made. The flasks are incubated at 28° to 30°C. Growth in the flasks will take place within two to four days, the paper becoming covered with a yellow or orange growth and showing rapid maceration. The culture is now transferred again to fresh silica plates, making a number of various dilutions from each flask. The highest dilution giving growth on the plate is then used for making further transfers. By transferring back to the flask and again to the plate, pure cultures may be finally obtained.

The yellow organism is very readily recognized as the *Spirochaeta cytophaga* of Hutchinson and Clayton. It decomposes the paper very rapidly, it does not grow on ordinary nutrient media and develops readily and abundantly on the silica gel plate. The identity of the orange-colored organism, which is a short rod, has not been established as yet. Both of these organisms are distinctly aerobic.

It can be demonstrated that, in addition to these aerobic organisms, there are also various other bacteria in nature which are capable of decomposing cellulose under aerobic and anaerobic conditions. Two grams of ground filter paper is added to about 100 gram portions of soil placed in tumblers, enough water is added to saturate the soil with an excess of about one inch of free water over the surface; the tumblers are placed in the incubator and kept at about 30°C. A considerable period of time will elapse before any manifestations of cellulose decomposition occur; only after one to two months incubation will gas begin to evolve accompanied by a sharp odor. This gas is found to consist of methane and CO_2. Cellulose decomposition which was at a standstill until this time will now begin rapidly. After all the cellulose has become decomposed, as indicated by the cessation of active "fermentation" and by actual quantitative cellulose determination, fresh portions of ground filter paper are added. Decomposition or "fermentation" will now set in immediately. This is due to the fact that normal soils do not contain the anaerobic bacteria or rather "the proper population required for anaerobic decomposition of cellulose," such as would no doubt be found in the case of sewage purification plants, and in peat or cranberry soils, always saturated with water. This population has to be developed in normal soils; a considerable period of time is required for this; once the population has become established, rapid cellulose decomposition, under anaerobic conditions, will take place. The organisms responsible for the decomposition of cellulose in this case are certain spore-forming bacteria. This can be readily demonstrated by inoculating the culture with sterile soil containing 1 per cent cellulose and 0.1 per cent NH_4NO_3 placed in flasks; some of the soil is brought to an optimum moisture content, adding sufficient water to make up 60 per cent saturation; to some of the soils an excess of water is added.

TABLE 1

CULTURE	MEDIUM = SOIL + CELLULOSE, STERILE	GROWTH
Aerobic* (heated to 70°C. for 10 minutes)	Optimum moisture	No visible growth
Aerobic, unheated	Optimum moisture	Abundant fungus development
Aerobic, heated	Excess moisture	No growth
Aerobic, unheated	Excess moisture	No growth
Anaerobic† (heated to 70°C. for 10 minutes)	Optimum moisture	No growth
Anaerobic, unheated	Optimum moisture	Scant fungus development
Anaerobic, heated	Excess moisture	Rapid cellulose "fermentation"
Anaerobic, unheated	Excess moisture	Rapid cellulose "fermentation"

* Aerobic = soil in which active cellulose decomposition has taken place under aerobic conditions.

† Anaerobic = soil containing excess water, in which cellulose "fermentation" has become very active.

The results indicate definitely that, under anaerobic conditions, a population has become established in the soil, which is capable of decomposing cellulose and which can resist heating to 70°C.

The population which causes the cellulose decomposition under aerobic conditions consists of fungi (Waksman and Skinner, 1926) and of bacteria, like *Sp. cytophaga*, both of which are destroyed by heating at 70°C. for 10 minutes.

When transfers are made from the soil covered with water, in which active cellulose "fermentation" is taking place, into flasks containing 1 gm. of filter paper, 25 cc. of the above nutrient medium and 150 cc. of water, growth will take place in a few days, manifested first by a uniform turbidity throughout the flask and accompanied by the production of gas and butyric acid, and finally also by the formation of a black precipitate which settles to the bottom. On transferring to aerobic silica plates, *Sp. cytophaga* may develop; in aerobic flasks the latter form may or may not develop; in "anaerobic" flasks, however, growth will take place. This growth is manifested by a rapid disintegration of the filter paper, formation of turbidity and often of a pellicle on the surface. At first gas formation takes place, but, on further transfer, the cultures show only cellulose disintegration without the formation of gas. These results as well as the results of other studies carried on in this laboratory tend to show that possibly both Omelianski (1908) and Kellerman and associates (1913) were correct in their interpretation of the phenomenon of cellulose decomposition by bacteria, namely that cellulose decomposition takes place under anaerobic conditions (Omelianski) and that we may have here a combined action of cellulose-decomposing organisms, which only decompose the paper, without gas formation, and butyric acid or other bacteria, which attack the products formed by the former, with the formation of acids and gases (Kellerman et al.). This does not exclude however, the possibility that there may be in nature organisms which are capable of decomposing celluloses under anaerobic conditions with the formation of acids and gases as shown by Khouvine (1923). In normal soils, however, cellulose decomposition is carried on entirely by filamentous fungi, as shown elsewhere (Waksman and Skinner), and by aerobic bacteria, as can be readily demonstrated by the silica plate method.

SUMMARY

1. A method is described which is very convenient for demonstrating the presence and nature of bacteria capable of decomposing celluloses.

2. This method, when combined with the proper dilutions, can also be used for determining the abundance of bacteria capable of decomposing celluloses, especially under aerobic conditions.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VISCOSITY OF BACTERIAL SUSPENSIONS

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I. INTRODUCTION AND THEORY

It is clearly established that many of the properties exhibited by bacteria suspended in non-nutrient menstrua are determined by physico-chemical mechanisms which are not directly associated with viability. The Brownian movement of bacteria and their reactions with reagents which affect it, the behavior of bacteria in an electrical field, the stability of bacterial suspensions and the flocculating effects of specific and non-specific substances—with respect to these and many other groups of phenomena the characteristics of bacteria are analogous to those of organic and inorganic matter in a state of fine dispersion. The dynamics of the reactions appear to be more closely related to the kinetics of surface energy than to the biochemical activities of protoplasm.

In a series of investigations which have been conducted in this laboratory we have undertaken to determine the extent to which the views of the late Prof. Jacques Loeb on the theory of colloidal behavior can be applied to account for the properties of bacteria in aqueous suspension, treating of the bacteria as though they were merely inert matter in the colloidal state. Our studies have been concerned principally with the so-called serological reactions, especially with acid, salt and serum agglutination of bacteria. In the course of these investigations one of us (I. S. F.) found that beyond a certain point the characteristics of bacterial suspensions could not be predicted from, nor could they be harmonized with, the views of Professor Loeb on colloidal behavior. Hence we undertook the series of experiments here reported to determine whether a readily measurable property of a bacterial suspension—its viscosity—varies under the influence of H-ion concentration, changes of bacterial concentration, etc., in the same way in which the viscosity of protein solutions has been demonstrated to vary under similar conditions by Professor Loeb.

From kinetic considerations Einstein (1906; 1911) derived a formula according to which the increased viscosity of a fluid which follows upon the introduction into it of a dispersed phase is directly proportional to the relative volume occupied by the dispersed phase. Thus:

$$\eta = \eta_0 (1 + \phi) \tag{1}$$

Later Einstein modified the formula by the introduction of a constant:

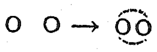
$$\eta = \eta_0 (1 + k\phi) \tag{2}$$

where η and η_0 are the viscosities of the colloidal suspension and of the dispersion medium respectively, k is constant and ϕ is the relative volume occupied by the dispersed particles. Einstein ascribed to k the value 2.5. He also pointed out that this formula describes the phenomena only under certain limiting conditions, i.e., when ϕ is relatively small and is considerably less than 0.5, and when the particles are, or approximate, smooth non-deformable spheres. In the application of this and similar equations to various colloidal suspensions the difficulty has been to determine the *volume* occupied by the dispersed particles so as not to rely upon their weight. We shall have occasion to indicate later that this difficulty is easily solved when bacteria are the dispersed particles. The values of k, determined by many workers, have commonly departed from Einstein's value (2.5) and have ranged from 1 to 4.75 (vide Hatschek, 1910–1913). Hatschek proposed to replace Einstein's constant by 4.5 and later his equation by the following:

$$\eta = \frac{\eta_0}{1 - \phi^{\frac{1}{3}}} \tag{3}$$

in which the increase of viscosity with increases of concentration of colloid is not linear. His conclusions have been criticized by numerous workers whose experimental findings demonstrate that the constant is not really constant, but is variable according to the nature of the solution studied.[1]

Smoluchowski (1916) has attempted to derive a generally applicable equation which would account for the increases of viscosity commonly observed upon the coagulation of a colloidal solution as well as for its normal viscosity. He arrived at the conclusion that the viscosity is increased by the electrical charge on the particles and that upon coagulation the viscosity must be increased at least 1.35 times if the particles remain even approximately spherical, because particles in approximate contact will appear to have increased in volume by a volume of adherent water (or other dispersing medium). Thus:



On these grounds Smoluchowski considered that upon coagulation the *apparent* volume occupied by the dispersed phase has increased although the *actual* volume has not changed.

More recently, Arrhenius (1917) has derived from Einstein's equation a logarithmic formula which has been widely used to describe the viscosity of a colloidal solution:

$$\log \eta - \log \eta_0 = \vartheta \phi \tag{4}$$

where η, η_0, and ϕ have the same significance as in (1), (2) and (3) above, and ϑ is a constant.

Loeb (1924) has found in his studies on the colloidal behavior of proteins that membrane potentials, osmotic pressure, swelling of gels and viscosity are dependent upon membrane equilibria of the type described by Donnan (1911). With respect to the viscosities of protein solutions his analysis may be considered, briefly, to postulate that the viscosity of a colloidal suspension (gelatin, albumin, etc., in water) is a function of water imbibition and swelling, that swelling is a function of the Donnan equilibrium and is dependent upon the degree of ionization of the suspended ampholyte. Hence, viscosity is at a minimum at the isoelectric point and rises to maximal values at H-ion concentrations above or below the pH of this point. Further, the addition of acid or alkali beyond the concentrations at which these maxima obtain causes diminutions in the viscosity. He has presented extensive experimental evidence which professes to demonstrate the validity of these hypotheses. We shall refer to his findings in greater detail below.

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[1] In our later references to "Hatschek's equation" it is Hatschek's modification of Einstein's equation, using the constant 4.5, to which we refer. Hatschek's own equation which involves the use of the cube root of the volume of dispersed phase we have found does not even approximate a description of the observed values. Hence calculations from it are not included except in the footnote to table 9.

We are not aware of any preceding researches on the viscosities of bacterial suspension. The experiments reported here were undertaken to determine the influences of H-ion concentrations upon the viscosities of such suspensions, working with a strain of *Bacterium coli* which has been utilized in extensive studies on the effects of ions upon viability (Winslow and Falk, 1923), upon the electrophoretic potentials of bacteria (Winslow, Falk and Caulfield, 1923; Winslow and Shaughnessy 1924) and upon the buffering properties of bacteria (Shaughnessy and Falk, 1924).

II. EXPERIMENTAL METHODS

To obtain bacterial suspensions, *Bact. coli* was grown for forty-eight hours in Kolle flasks at 37°C.; the bacteria were washed off in a few cubic centimeters of distilled water by gently rotating the flask, and were shaken to break up clumps. For experiments 1 to 3 described below the suspension was diluted in distilled water and the pH adjusted without further treatment. For experiments 4 to 7 the cells were thrown down and washed four times by centrifugalization, resuspended in distilled water and the pH adjusted with HCl or NaOH. The concentrations of the suspensions were determined by comparisons with barium sulphate turbidity standards. The pH was adjusted by adding the amount of acid or alkali, determined by titrating a portion of the suspension, viz., to 5 cc. two to four drops of suitable indicator and acid or alkali were added, with shaking, until a color standard was matched. Standard color plates were used for comparison (Clark, 1922). The suspensions were shaken to distribute the acid or alkali and to saturate or equilibrate the buffer capacity of the cells (Winslow and Shaughnessy, 1924). The pH of each suspension was again determined after completing the viscosity measurement.

Ostwald viscometers were used for measurements. They were cleaned before each measurement by drawing through them potassium dichromate cleaning solution, soaking in this fluid for an hour or more, washing thoroughly in tap water, rinsing with about 50 cc. of distilled water, washing with 95 per cent alcohol and anhydrous ether and drying with filtered air. Viscometer tubes containing 5 cc. of water, or of any suspension to be measured, were immersed in a water bath of a capacity such that with a mechanical stirring device it could be kept at 25 ± 1°C. for the duration of a series of measurements. When the temperature in the tubes came to that of the bath the temperature was read to 0.1 of a degree and recorded, the fluid was drawn up into the bulb and the time of outflow of a constant volume of fluid through the viscometer capillary was read and recorded to 0.2 of a second. In each case the measurement was repeated once or twice and when the reading varied slightly, the minimum was recorded. If the time varied more than two seconds (except in the case of suspensions at pH 13 which were heavily flocculent and sometimes caused temporary stoppage of the capillary) the suspension was discarded and another prepared. A variation of two seconds gives a percentage error of from 1 per cent for the heaviest suspensions to 2 per cent for the least concentrated. This agrees with findings from three attempts to determine the experimental error.

With each tube duplicate measurements on dust-free distilled water gave the same reading. The tubes used had a time of outflow from 80.6 to 90.8 seconds. From these readings the time of outflow for each tube for each tenth of a degree between 24° and 26°C. was calculated. A 10 cc. pycnometer was used for density determinations.

The organism used, a water viable strain of *Bacterium coli*, was chosen because it is non-pathogenic, grows readily on nutrient agar, is easily removed from the surface of the medium and hence can be obtained without difficulty in the large quantities necessary for these experiments; because it can be suspended in distilled water and the effect of salt or broth on pH or viscosity ruled out; because the acid and alkaline isolectric points (pH 2.7 to 3.0 and pH 13.5 to 13.8) are known (Winslow and Shaughnessy, 1924; Eggerth, 1923); and because it produces no appreciable capsular material to complicate viscosity measurements.

Suspensions were examined both macroscopically and microscopically for agglutination. The results are recorded with the tabular data.

Viscosity ratio (viscosity of suspension / viscosity of water) was determined by the formula suggested by Ostwald (1910):

$$\frac{\eta}{\eta_0} = \frac{\rho\, t}{\rho_0\, t_0}$$

where η is the viscosity of the suspension; η_0 is the viscosity of water at the temperature of the experiment; ρ is the density of the suspension; ρ_0 is the density of water; and t and t_0 are the time of outflow for the suspension and water respectively.

When one of the suspensions used in experiment 3 was centrifuged at high speed until the cells were tightly packed in the bottom of the tube, a viscosity measurement upon the dispersion medium revealed that it had a somewhat higher time of flow than water. An attempt to calculate the time of flow for the same concentration of cells suspended in water was then made. The difference between the time of flow for the dispersion medium and the time of flow for water at that temperature was deducted from the time of flow for the suspension at the same temperature.

III. EXPERIMENTAL FINDINGS

By reference to table 1 and figure 1 it can be seen that with a suspension containing one billion bacteria per cubic centimeter changes in pH between 2.8 and 9.2 were practically without measurable effect upon viscosity except at pH = 3.6 where a slight but significant rise is observed. The results obtained with suspensions containing 3.5 and 8.3 billion bacteria per cubic centimeter prepared in a similar manner (unwashed bacteria) are given in tables 2 and 3 and in figures 2 and 3.

In tables 2 and 3 and in figures 2 and 3 (as was the case in table 1 and figure 1 above) it is clearly evident that within the range of pH studied (1.4 to 9.0) no significant changes in viscosity are evident except at pH = 3.8. In each of these three experiments with unwashed bacteria suspended in distilled water (1, 3.5 and 8.3 billions per cubic centimeter) acid agglutination of the bacteria was observed at that pH.

TABLE 1

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water.
Final suspension, 1 billion per cubic centimeter.
Measurements at 25 ± 1°C.
Agglutination at pH = 3.6.
Inclusion of densities in calculations changes results less than 0.1 per cent.
Time of outflow for dispersion medium same as for water.

pH	η(obs.)	η_0(obs.)	η(rel) (calc.)
2.8	85.2	84.6	1.007
3.6	96.6	89.5	1.079
4.6	88.4	86.4	1.023
5.4	84.6	83.2	1.017
6.4	83.8	82.4	1.017
6.6	87.2	85.6	1.019
6.8	87.8	86.0	1.021
7.2	80.2	79.8	1.005
7.8	85.2	84.0	1.014
8.2	80.8	80.0	1.010
8.4	80.6	80.0	1.007
9.2	91.2	90.4	1.009

Further experiments were then conducted with *washed* bacteria exposed to H-ion concentrations extending over a wider range, i.e., pH = 0 to pH = 14.0. The results with suspensions containing 5, 10, 17 and 50 billions per cubic centimeter successively are presented in tables 4 to 7 and in figure 4.

TABLE 2

Bacteria grown on nutrient agar 48 hours at 37°C.; suspended in distilled water.
Final suspension, 3.5 billion per cc.
Measurements at 25 ± 1°C.
Agglutination at pH = 3.8.
Densities of dispersion medium and water differed within the limits of experimental error.
Density of suspension, 1.000 at 25°C.
Time of outflow of dispersion medium same as for water.

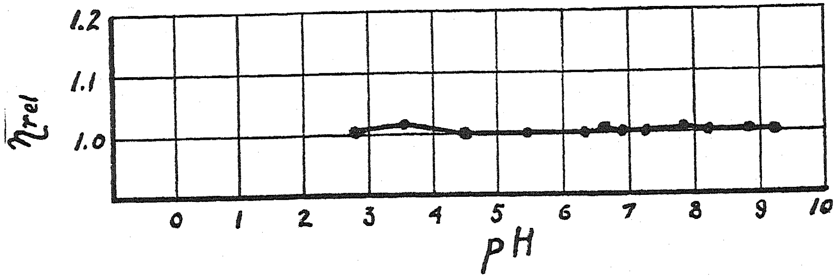
pH	t (OBS.)	t_0 (OBS.)	t/t_0 (CALC.)	η/η_0 (CALC.)
3.4	91.6	84.4	1.085	1.088
3.8	103.2	88.6	1.165	1.168
5.6	84.6	80.2	1.055	1.058
6.8	91.6	85.6	1.070	1.072
7.0	96.6	88.8	1.088	1.091
7.8	91.6	85.4	1.073	1.076
8.0	93.0	87.2	1.066	1.069
8.4	91.0	83.2	1.094	1.097

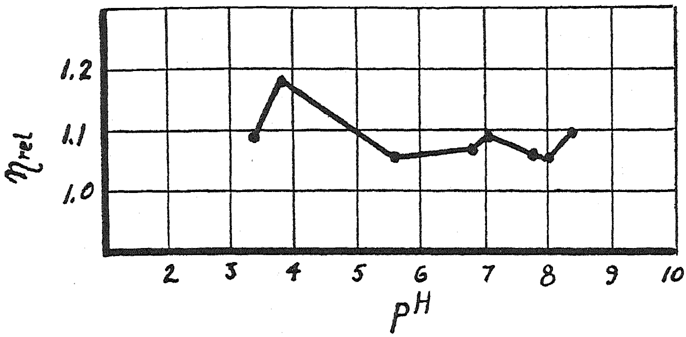
TABLE 3

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water.
Final suspension, 8.3 billion per cc.
Measurements at 25 ± 1°C.
Agglutination at pH = 3.8.
Density of washed cells suspended in water at 25°C.,1.000.
Values in the last column are calculated as explained in the text.

pH	t (OBS.)	t_0 (OBS.)	t/t_0 (CALC.)	η/η_0 (CALC.)
1.4	96.0	85.4	1.124	1.028
1.8	92.8	83.0	1.118	1.022
2.4	97.4	83.0	1.173	1.078
3.8	115.2	85.0	1.355	1.260
4.0	102.0	87.8	1.161	1.066
5.2	102.4	90.4	1.133	1.036
6.2	89.2	80.2	1.112	1.017
7.0	97.2	84.2	1.154	1.059
7.8	96.2	84.6	1.137	1.041
9.0	98.4	86.0	1.149	1.048

From tables 4 to 7 it is clearly evident that increases in viscosity occur only at pH values near 3.8 and 13.0, the H-ion concentrations at which agglutination of the bacteria is recorded.





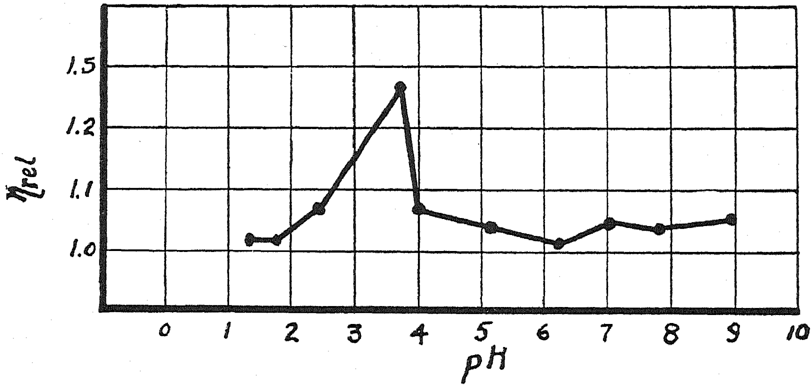


FIG. 1. THE INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VISCOSITY OF BACTERIUM COLI
1 billion bacteria per cubic centimeter

FIG. 2. THE INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VISCOSITY OF BACTERIUM COLI
3.5 billion per cubic centimeter

FIG. 3. THE INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VISCOSITY OF BACTERIUM COLI
8.3 billion per cubic centimeter

The increases in viscosity are far more marked in the zone of the alkaline than in the zone of the acid isoelectric point. Since the work of Michaelis in 1911 (1911) it has been known that ~~bacteria und~~ergo spontaneous agglutination at suitable acidities; and more recent investigations (Northrop and DeKruif, 1922) have demonstrated that the zone of acid agglutination centres about the isoelectric point. That bacteria will also manifest an alkaline isoelectric point was predicted by Shaughnessy and Falk (1924) from experimental findings on the buffering capacities of bacteria at various H-ion concentrations and were actually demonstrated by Winslow and Shaughnessy (1924).

TABLE 4

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water; washed four times.
Final suspension, 5 billion per cc.
Measurements at 24.5 ± 0.5°C.
Agglutination at pH = 3.4 – 3.8 and pH = 13.
~~At pH = 14~~ suspension slimy, most of cells dissolved.
Density at 25°C. – pH = 0, 1.018; pH = 1, 1.000; pH = 2–12, 0.998; pH = 13, 1.003; pH = 14, 1.046.
Viscosity at pH = 0–1 and 12–14 corrected for viscosity of acid and alkali by subtracting from the total viscosity the measured viscosity of the acid or alkali ~~less one.~~

pH	η (OBS.)	η_0 (OBS.)	η (REL) (CALC.)	η (REL) (CORRECTED)
0.0	93.0	87.1	1.068	1.036
1.0	89.2	86.1	1.035	1.033
2.0	85.8	81.4	1.054	
~~3.4~~	90.2	86.9	1.038	
3.8	89.0	85.3	1.043	
4.0	87.0	84.5	1.029	
5.2	87.2	85.1	1.025	
6.4	81.8	80.6	1.015	
~~7.6~~	90.2	88.1	1.024	
8.8	92.8	90.5	1.025	
10.0	86.6	83.9	1.032	
11.0	97.2	92.3	1.053	
12.0	90.0	85.7	1.050	1.048
~~13.0~~	99.1	82.4	1.203	1.188
14.0	112.5	91.6	1.229	1.047

Our findings, recorded in these tables, also indicate a zone of alkaline agglutination centering about the position of the alkaline isoelectric point of *Bact. coli.* They show, further, that with increasing concentrations of bacteria in aqueous suspensions the viscosity is increased somewhat at all pH values and rises especially in the zones of agglutination about the acid and alkaline isoelectric points. In figure 4 we have brought together the data given separately in tables 4 to 7 prepared from the experiments with washed bacteria. They show that in general pH affects viscosity only at the two isoelectric points and that viscosity increases with increasing concentration of bacteria.

TABLE 5

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water; washed four times.

Final suspension, 10 billion per cc.

Measurements at 24.1 ± 0.1°C.

Agglutination at pH = 3.2 – 4.0 and pH = 13.

At pH = 14 suspension slimy, part of cells dissolved, part clumped.

Density at 24°C. – pH = 0, 1.020; pH = 1, 1.002; pH = 2– 12, 1.000; pH = 13, 1.005; pH = 14, 1.048.

Viscosity at pH = 0 – 1 and 12–14 corrected for viscosity of acid and alkali by subtracting from the total viscosity the measured viscosity of the acid or alkali less one.

pH	η (OBS.)	η_0 (OBS.)	η (REL) (CALC.)	η (REL) (CORRECTED)
0.0	96.2	89.2	1.078	1.047
1.0	87.4	81.7	1.070	1.068
1.6	91.0	85.9	1.059	
2.2	90.8	85.3	1.065	
3.2	89.6	84.1	1.065	
4.0	95.2	87.4	1.089	
5.0	86.0	81.9	1.050	
6.2	95.2	89.8	1.060	
7.0	95.2	89.8	1.060	
8.0	84.4	81.5	1.036	
9.0	92.4	85.3	1.083	
10.0	96.2	89.8	1.071	
11.0	95.0	81.9	1.160	
12.0	98.6	86.3	1.143	1.141
13.0	110.6	92.8	1.192	1.169
14.0	112.3	91.3	1.230	1.044

In table 8 the data from all the experiments are summarized in average relative viscosities for each unit range of pH, for each concentration of bacteria studied and for all concentrations combined. From the average data in this table we have plotted separately the effect of concentration of bacteria upon viscosity for each unit range of pH (figs. 5 to 19) and the effect

TABLE 6

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water; washed four times.

Final suspension, 17 billion per cc.

Suspension kept overnight in the ice box before measurements were made. Became slightly alkaline (pH = 8).

Agglutination slight at pH = 3.8, pronounced at pH = 4.6 and pH = 13.

Measurements at 25 ± 1°C.

Density at 25°C., pH = 0, 1.021; pH = 1, 1.003; pH = 2–12, 1.001; pH = 13, 1.006; pH = 14, 1.049.

At pH = 14 suspension slimy, some of cells dissolved, remainder clumped.

pH	η (OBS.)	η_0 (OBS.)	η (REL) (CALC.)	η (REL) (CORRECTED)
0.0	111.7	92.9	1.202	1.099
1.0	98.9	88.3	1.120	1.118
2.0	94.5	80.7	1.171	
2.4	95.7	84.5	1.133	
3.4	98.3	85.1	1.155	
3.6	97.1	84.5	1.149	
3.8	104.1	90.3	1.153	
4.0	100.7	87.7	1.148	
4.2	97.9	85.3	1.148	
4.6	96.1	79.7	1.206	
4.8	94.3	83.3	1.132	
5.0	87.9	79.9	1.100	
6.2	96.1	87.9	1.092	
7.0	94.7	85.3	1.110	
8.0	100.9	90.7	1.112	
8.8	93.5	84.3	1.107	
10.2	95.7	85.7	1.117	
11.0	96.1	87.7	1.096	
12.0	97.9	82.1	1.192	1.100
13.0	134.6	84.2	1.599	1.575
14.0	127.6	91.3	1.398	1.211

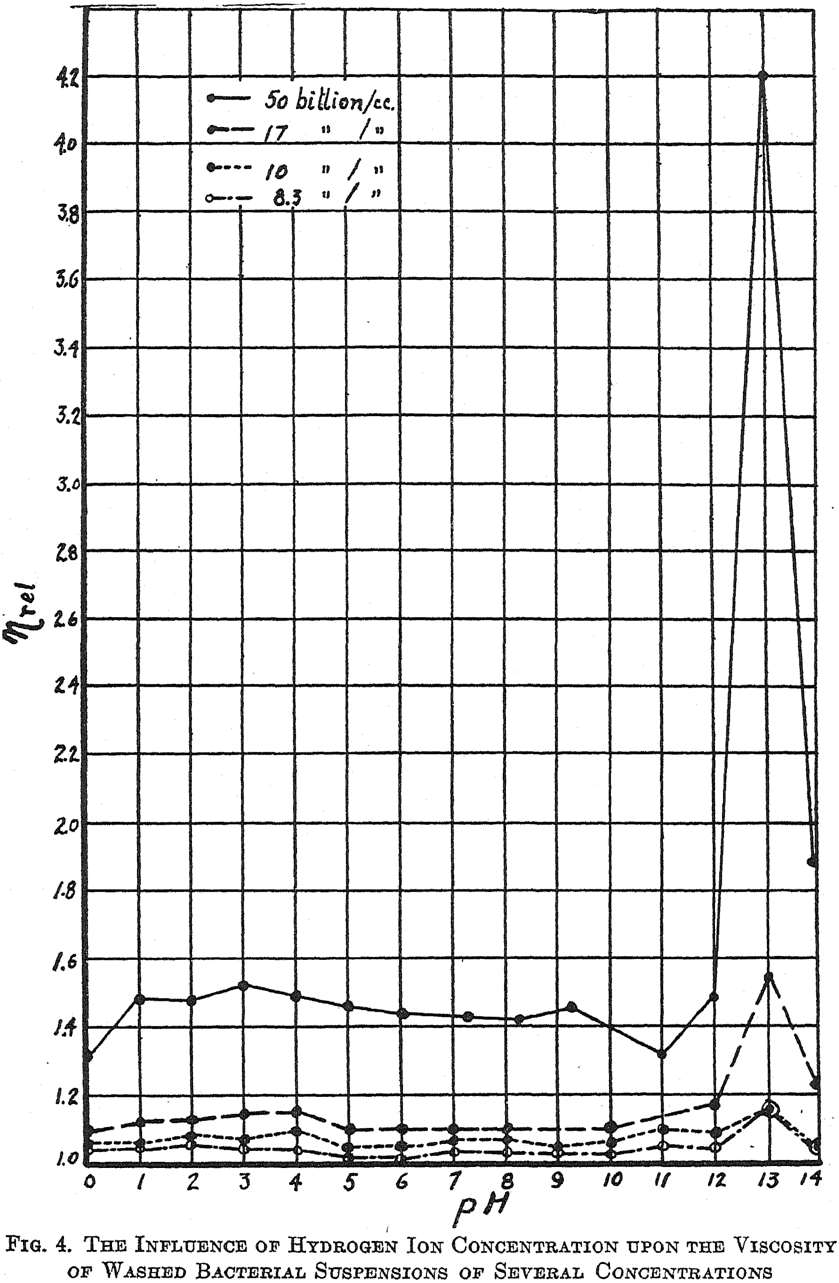
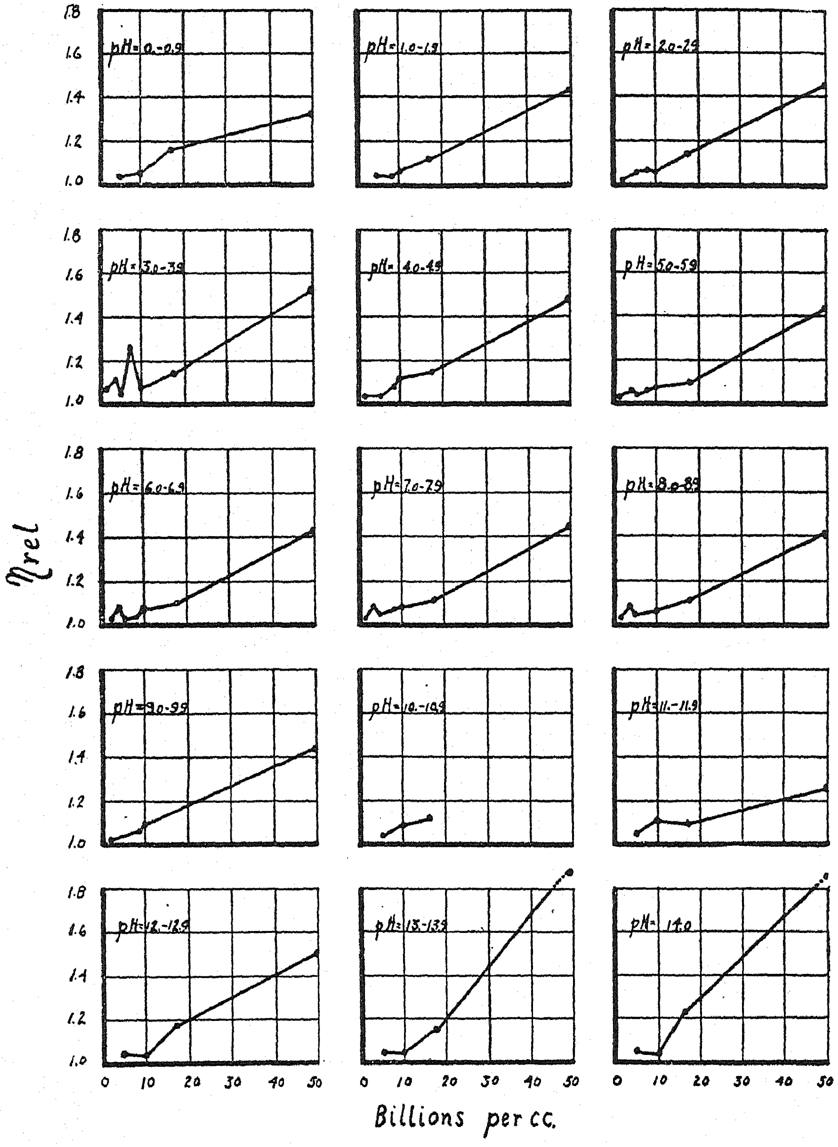


FIG. 4. THE INFLUENCE OF HYDROGEN ION CONCENTRATION UPON THE VISCOSITY OF WASHED BACTERIAL SUSPENSIONS OF SEVERAL CONCENTRATIONS



FIGS. 5 TO 19. THE RELATIONS BETWEEN BACTERIAL CONCENTRATION AND VISCOSITY AT VARIOUS HYDROGEN ION CONCENTRATIONS

TABLE 7

Bacteria grown on nutrient agar forty-eight hours at 37°C.; suspended in distilled water; washed four times.

Final suspension, 50 billion per cubic centimeter.

Agglutination at pH = 2.6 – 3.6 and pH = 13.

Measurements at 25 ± 1°C.

Density at 25°C., pH = 0, 1.027; pH = 1, 1.009; pH = 2 – 12, 1.007; pH = 13, 1.012; pH = 14, 1.055.

Viscosity at pH = 0 – 1 and 12 – 14 corrected for viscosity of acid and alkali by subtracting from the total viscosity the measured viscosity of acid or alkali less one.

Suspension in normal alkali slimy. Part of bacteria dissolved, part in amorphous clumps.

Suspension at pH = 13 heavily flocculent. Clumps, several times as large as those at the acid isoelectric point, clung to walls of capillary causing a disproportionate increase in the time of flow.

pH	η (OBS.)	η_0 (OBS.)	η (REL.) (CALC.)	η (REL.) (CORRECTED)
0.0	118.9	85.8	1.386	1.353
1.0	129.2	87.9	1.469	1.467
2.0	121.6	85.3	1.426	
2.6	125.2	83.5	1.499	
3.0	128.2	85.3	1.503	
3.2	133.2	87.7	1.518	
3.6	127.8	84.3	1.516	
3.8	125.2	84.3	1.485	
4.4	132.8	89.2	1.489	
4.6	125.4	85.5	1.467	
4.8	131.0	90.1	1.454	
5.2	120.4	83.5	1.442	
6.2	119.8	83.9	1.429	
7.0	114.0	79.6	1.432	
8.0	113.2	80.0	1.415	
9.2	124.0	85.7	1.447	
11.0	115.8	87.3	1.326	
12.0	122.0	79.6	1.535	1.533
13.0	357.6	83.8	4.268	4.185
14.0	173.7	86.3	2.013	1.825

of pH upon viscosity for all concentrations combined (fig. 20).[2] In figure 20 we have also included (as a dotted line) a curve

[2] By computing the percentage change in the height of all the curves for each pH unit range and using these figures the curves for the experiments presented in tables 1 to 3 were extrapolated to the pH limits used in the later experiments reported in tables 4 to 7. When these computed values were averaged with the

representing a hypothetical extrapolation of viscosity at various pH values based upon Loeb's findings with gelatin. This hypothetical curve is based upon the experimental findings reported by Loeb at pH values in the range 1.0 to 6.0 and upon the assumptions: (*a*) that in more alkaline zones the curves are continuous with those in acid zones; and (*b*) that the discovery of a second (alkaline) isoelectric point at or near pH 13.0 would demand the occurrence of a second minimum in the viscosity

TABLE 8

Relative viscosity averages for unit ranges of pH

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	pH							
	0–0.9	1–1.9	2–2.9	3–3.9	4–4.9	5–5.9	6–6.9	7–7.9
1.0			1.007	1.079	1.023	1.017	1.019	1.010
3.5				1.128		1.058	1.072	1.083
5.0	1.036	1.033	1.054	1.040	1.029	1.025	1.015	1.024
8.3		1.028	1.078	1.260	1.066	1.036	1.017	1.050
10.0	1.047	1.064	1.065	1.065	1.089	1.050	1.060	1.060
17.0	1.099	1.118	1.152	1.152	1.159	1.100	1.092	1.110
50.0	1.353	1.467	1.462	1.506	1.437	1.442	1.429	1.432
Average..	1.139	1.142	1.137	1.176	1.134	1.104	1.101	1.110

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	pH							
	8–8.9	9–9.9	10–10.9	11–11.9	12–12.9	13–13.9	14	
1.0	1.009	1.009						
3.5	1.083							
5.0	1.025		1.032	1.053	1.048	1.088	1.047	
8.3		1.048						
10.0	1.036	1.083	1.071	1.160	1.141	1.169	1.044	
17.0	1.110		1.117	1.096	1.190	1.675	1.211	
50.0	1.415	1.447		1.326	1.533	4.185	1.825	
Average..	1.113	1.147	1.073	1.159	1.228	2.029	1.182	

---

measured values the net result was—except for a lowering of the peak at pH = 13—the same as that given in figure 20. Hence, the data computed in this manner were not included.

curve. This curve is introduced merely to emphasize our regular observation of a *maximum* instead of a *minimum* viscosity value at or near an isoelectric point.

In connection with the comparison made between our findings and the type of curve which would be obtained if Loeb's findings (extrapolated) were applicable to bacterial suspensions (figure 20) it must be pointed out that our suspensions remained in the water bath for some time before the measurements were made, whereas Loeb's solutions of gelatin were measured for viscosity immediately after being brought to the desired pH values and the desired temperature. Loeb found that when gelatin solutions were kept at 20°C. for one hour before measurements were made viscosity increased near the isoelectric point. He attributed this to the formation of submicroscopic particles of jelly and he believed that while at more acid reactions the formation of these micellae is prevented yet the acid causes the particles already present to swell. He found that the viscosity ratio of

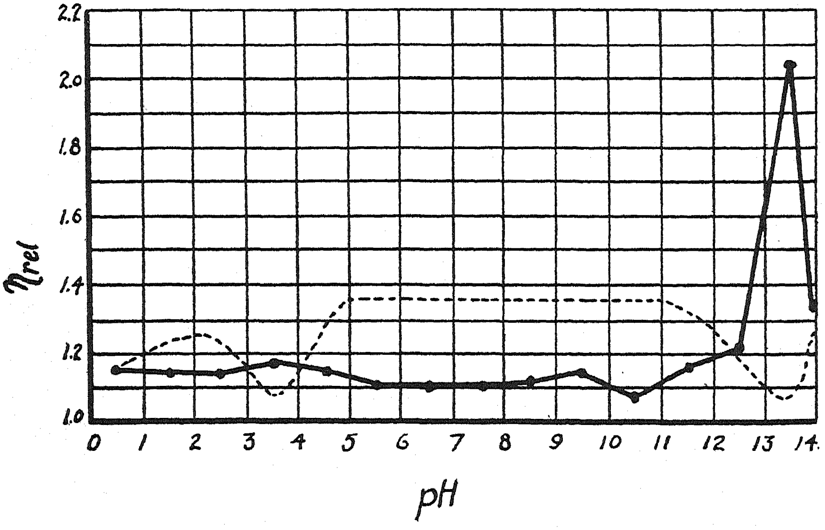


FIG. 20. THE RELATIONS BETWEEN VISCOSITY AND HYDROGEN ION CONCENTRATION

The solid line is an average from all experiments, disregarding the different concentrations of bacteria. The dotted line is a hypothetical curve based on an extrapolation of Loeb's findings with gelatin (disregarding ordinates).

2 per cent gelatin sulphate solution increased from 3 to 7 after standing near the isoelectric point for 10 or 15 minutes.[3] The time factor is not strictly applicable to the influence of acid agglutination on viscosity of bacterial suspensions since, although several minutes are required for complete agglutination, a relatively large proportion of the cells are clumped immediately. We shall discuss this point again below.

It is indicated in figures 5 to 19 that viscosity of bacterial suspensions is not an arithmetic linear function of the concentration of the disperse phase and hence that neither Einstein's equation nor Hatschek's modification of it will fit the observations. We propose to show more succinctly that this is the case and that Arrhenius' equation gives a very good agreement with our findings.

We may, at this point, re-state the three equations used in our calculations:

Equation of Einstein

$$\eta = \eta_o\ (1 + 2.5\ \phi)$$

Equation of Einstein, modified by Hatschek

$$\eta = \eta_o\ (1 + 4.5\ \phi)$$

Equation of Arrhenius

$$\log \eta - \log \eta_o = \vartheta\ \phi$$

where η = viscosity of the colloidal suspension,
η_0 = viscosity of the menstruum,
ϕ = relative volume occupied by the dispersed colloid,
ϑ = a constant.

Taking for the size of *Bacterium coli* average values of length and diameter and considering it as a right cylinder ($2.5\mu \times 0.5\mu$) we have calculated the volume (in cubic centimeters) of one billion cells and from that arrived at the value of ϕ of Arrhenius' equation for five, ten, seventeen and fifty billion bacteria per cubic centimeter. We then found from tables 4 to 7 the average relative viscosity of each suspension (all pH values combined), calculated the value of the constant ϑ, and, using this and Einstein's and Hatschek's constants, calculated the viscosity with each of the three equations. Table 9 and figure 21 show that

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[3] Some years ago, Gokun (Zeitschr. Chem. Ind. Koll., 1908, **3**, 84) reported similarly that the minima in viscosity curves for gelatin + electrolytes disappear after the lapse of several hours.

TABLE 9

Relative viscosities—measured and calculated from the theoretical equations of Arrhenius, Einstein and Hatschek

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	η (OBS.) AVERAGE	η_0 (OBS.) AVERAGE	η (REL) $2 \div 3$	VOLUME OF BACTERIA (ϕ)	η (REL) — CALCULATED BY		
					Arrhenius' equation ($\vartheta = 8.0$) (calc.)	Einstein's equation ($K = 2.5$)	Hatschek's equation* ($K = 4.5$)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
				cc.			
5	90.1	86.1	1.046	0.00246	1.046	1.006	1.011
10	93.4	86.5	1.079	0.00491	1.096	1.012	1.022
17	99.4	85.8	1.158	0.00835	1.166	1.021	1.038
50	136.9	84.9	1.612	0.02460	1.573	1.062	1.111

* By "Hatschek's equation" in this and subsequent tables we refer to Hatschek's modification of Einstein's equation. Using Hatschek's equation $\left(\eta = \frac{\eta_0}{1 - \phi^{\frac{1}{3}}}\right)$ we obtain calculated values so far from the measured values of η rel that they are not included in the Tables. Thus, for the data in this table the calculated values of η rel are 0.865; 1.205; 1.255 and 1.410.

TABLE 10

Relative viscosity (measured and calculated) at pH = 6 to pH = 8

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	η (OBS.) (AVERAGE)	η_0 (OBS.) (AVERAGE)	η (REL) $2 \div 3$	VOLUME OF BACTERIA (ϕ)	η (REL) CALCULATED BY		
					Arrhenius' equation ($\vartheta = 6.0$)	Einstein's equation ($K = 2.5$)	Hatschek's equation ($K = 4.5$)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
				cc.			
5	86.0	84.4	1.019	0.00246	1.035	1.006	1.011
10	91.6	87.0	1.053	0.00491	1.070	1.012	1.022
17	97.2	88.0	1.104	0.00835	1.122	1.021	1.038
50	115.7	81.2	1.425	0.02460	1.405	1.062	1.111

Arrhenius' equation gives viscosity values which closely agree with the measured values and that as the concentration of the suspension increases the values calculated from Einstein's and Hatschek's equations lead to increasingly impossible results.

In order to determine more nearly the actual value of the constant ϑ of Arrhenius' equation we repeated the calculations, using

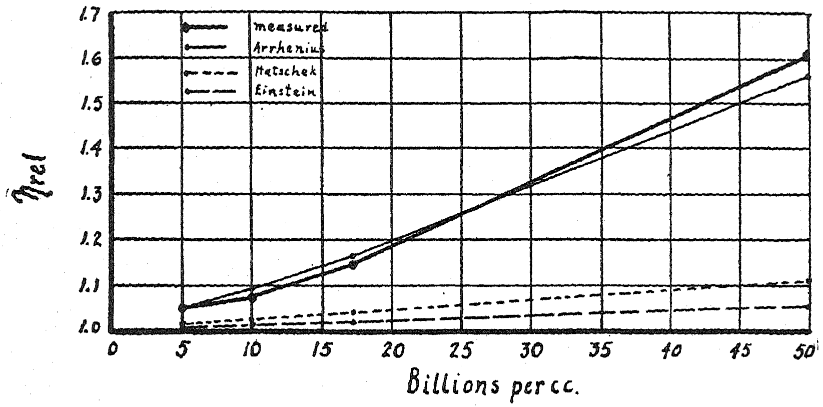

FIG. 21. THE RELATIONS BETWEEN BACTERIAL CONCENTRATION AND VISCOSITY (MEASURED AND CALCULATED), ALL pH VALUES AVERAGED

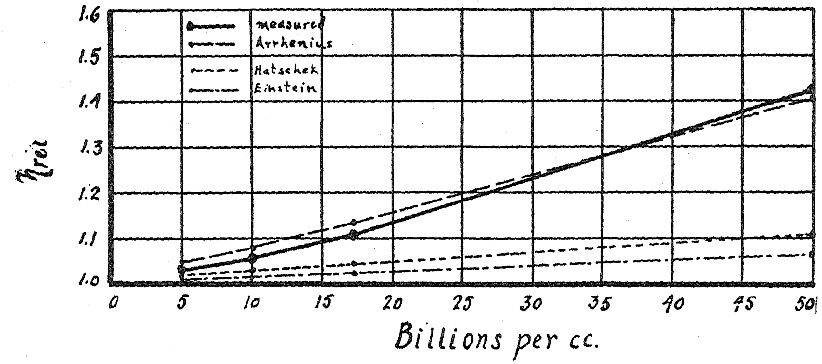

FIG. 22. THE RELATIONS BETWEEN BACTERIAL CONCENTRATION AND VISCOSITY (MEASURED AND CALCULATED), pH = 6.0 TO pH = 8.0

only the viscosities measured at pH values near neutrality. These are presented in table 10 and in figure 22. It is evident from the data in table 10 and the curves in figure 22 that with the calculated value $\vartheta = 6.0$ there is close agreement between the observed and the calculated viscosities for suspensions of

Bact. coli at pH values between 6.0 and 8.0. When the same value for the constant ($\vartheta = 6.0$) was used to calculate viscosity at pH values near either of the isoelectric points (acidic, pH = 3.0 to 4.0: alkaline, pH = 13.0) it was found to give values significantly lower than those found by experiment. For viscosities in the pH range 3.0 to 4.0 the value of ϑ was calculated to be approximately 7.2, and at pH = 13.0 approximately 23.6. From the data calculated with these values for the constants

TABLE 11

Relative viscosity (measured and calculated) at pH = 3 to pH = 4

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	η (OBS.) (AVERAGE)	η_0 (OBS.) (AVERAGE)	η (REL) 2 ÷ 3	VOLUME OF BACTERIA (ϕ)	η (REL) CALCULATED BY			
					Arrhenius' equation		Einstein's equation ($K = 2.5$)	Hatschek's equation ($K = 4.5$)
					($\vartheta = 6.0$)	($\vartheta = 7.2$)		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
				cc.				
5	86.7	85.5	1.014	0.00246	1.035	1.042	1.006	1.011
10	92.4	85.7	1.078	0.00491	1.070	1.085	1.012	1.022
17	100.1	86.9	1.152	0.00835	1.122	1.148	1.021	1.038
50	128.6	85.4	1.506	0.02460	1.405	1.504	1.062	1.111

TABLE 12

Relative viscosity (measured and calculated) at pH = 13)

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER)	η (OBS.)	η LESS η N/10 ALK. (OBS.)	η_0 (OBS.)	η (REL) 3 ÷ 4	VOLUME OF BACTERIA (ϕ)	η (REL) CALCULATED BY			
						Arrhenius' equation		Einstein's equation ($K = 2.5$)	Hatschek's equation ($K = 4.5$)
						($\vartheta = 6.0$)	($\vartheta = 23.6$)		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
					cc.				
5	99.1	97.1	82.4	1.888	0.00246	1.035	1.143	1.006	1.011
10	110.6	108.5	92.8	1.169	0.00491	1.070	1.306	1.012	1.022
17	134.6	132.8	84.2	1.575	0.00835	1.122	1.574	1.021	1.038
50	357.6	350.7	83.8	4.185	0.02460	1.405	3.807	1.062	1.111

curves are obtained which approximate closely to the curve drawn from viscosity values obtained by experiment (tables 11 and 12; figs. 23 and 24).

At this point we may turn to a consideration of the mechanisms involved in the modification of viscosity by changes in hydrogen ion concentration. Inasmuch as our experiments were conducted with a colloid-like suspension in which the dispersed particles (bacteria) differ from ordinary colloids in that they

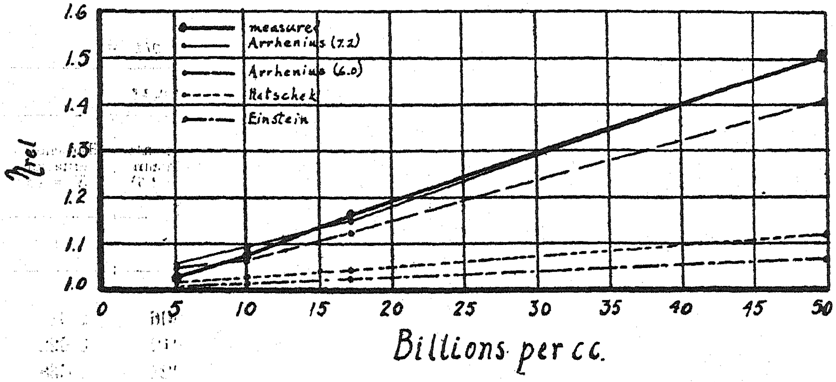

FIG. 23. THE RELATIONS BETWEEN BACTERIAL CONCENTRATION AND VISCOSITY (MEASURED AND CALCULATED), pH = 3.0 TO pH = 4.0

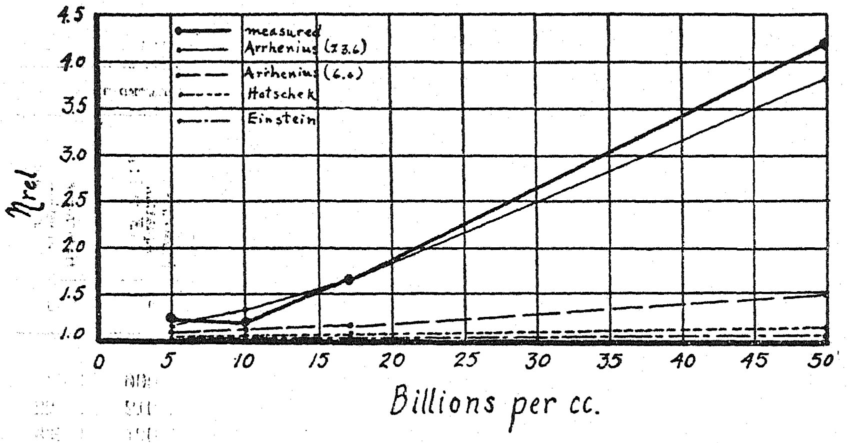

FIG. 24. THE RELATIONS BETWEEN BACTERIAL CONCENTRATION AND VISCOSITY (MEASURED AND CALCULATED) AT pH = 13.0

do not, under ordinary experimental conditions, change in their state or degree of dispersion, it might appear to follow that an equation like that of Arrhenius should describe viscosity at one pH as well as at another. We have pointed out that our experimental findings can be adequately described by this equation without changes in the value of the constant involved at all pH values except those near the two isoelectric points of the bacteria. At those points the viscosity is increased and the Arrhenius constant (ϑ) must be increased to obtain an approximation between measured and calculated values of viscosity. However, from certain *à priori* considerations, it appears that this cannot be considered a valid procedure nor can it lead to any advancement of our knowledge of colloidal behavior. On the other hand, it is more reasonable to assume that if the thermodynamic considerations which underlie the deduction of Arrhenius' equation are both necessary and sufficient the "constant" is truly constant. When, with a given set of conditions (as in our experiments at or near the isoelectric points), the viscosity values calculated from the equation do not approximate the measured values it may be more reasonable to consider that the value of ϕ (the relative volume occupied by the disperse phase) rather than the value of ϑ, the constant, has changed. That there is good grounds for such a consideration has already

TABLE 13

The values of ϕ (calculated) when ϑ of Arrhenius' equation is equal to 6.0

CONCENTRATION (BILLIONS PER CUBIC CENTIMETER	VOLUME OF BACTERIA (ϕ)	η (REL) AT pH = 3 – 4 (TABLE XI)	VOLUME OF DISPERSE PHASE AT pH = 3 – 4 (ϕA)	pH = 3 – 4 RATIO $\frac{\phi A}{\phi}$	η (REL) AT pH = 13 (TABLE XII)	VOLUME OF DISPERSE PHASE AT pH = 13 (ϕB)	pH = 13 RATIO $\frac{\phi B}{\phi}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	cc.		*cc.*			*cc.*	
5	0.00246	1.014	0.00101	0.41	1.188	0.01247	5.07
10	0.00491	1.078	0.00544	1.11	1.169	0.01130	2.30
17	0.00835	1.152	0.01026	1.23	1.575	0.03288	3.94
50	0.02460	1.506	0.02964	1.20	4.185	0.10360	4.21

been indicated in the remarks (p. 99) concerning Smoluchowski's work and, in connection with each experiment, concerning the occurrence of agglutination of the bacteria in the two zones of pH in which the viscosity rises. It is significant to recall that when bacteria (or other colloid-like particles) agglutinate or "coagulate" the clumped aggregate and the dispersion medium occluded in it behave as a unit, not only when there is streaming in the fluid due to mechanical forces but even when there is opposite streaming of the aggregated particles and of the menstruum in an electrical field (cataphoresis). These considerations lead to the conclusion that the volume occupied by the dispersed phase is *apparently* increased by the volume of occluded water although the *actual* volume occupied by the particles (bacteria) is unchanged. In table 13 are presented the data which show what the apparent relative volume of the dispersed phase (ϕ) would be near the two isoelectric points if the value of the constant in Arrhenius' equation be considered the same (6.0) at all pH values.[4]

In this table it is seen that the ratio of the apparent volume of the dispersed phase near the acid isoelectric point to the volume calculated directly from the dimensions and the concentrations of *Bact. coli* $\left(\frac{\phi\ A}{\phi}\right)$ is about 1.2. The ratio for the suspension containing 5 billions per cubic centimeter is less than one. This is probably due to experimental error. It is also seen that near the alkaline isoelectric point the ratio $\left(\frac{\phi\ B}{\phi}\right)$ varies from 2.3 to 5.07, with an average value of 3.88.

We have conducted a series of measurements to determine the relative volumes occupied by the clumps of bacteria which are observable under the microscope in suspensions at pH = 3.6 to 3.8 and at pH = 13 and the actual volume occupied by the bacteria in the clumps. The gross volumes of the clumps (bacteria plus occluded water) were calculated from average measurements of the outside dimensions of the clumps and the volumes of the bacteria in the clumps from the average number of bacteria in each average clump and from the known average volume of each bacterium. In the acidulated solutions, the ratio: $\frac{\text{volume of clump}}{\text{volume of bacteria}}$ is approximately 67; in the alkalinized solutions the ratio is approximately 87. Hence, it follows that if even a small proportion of the bacteria in a suspension are in clumps the apparent volume of the dispersed phase is considerably increased.

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[4] Disregarding the probable effect of mechanical hindrance to flow in the case of the suspension of 50 billion bacteria per cubic centimeter at pH = 13.0.

Inasmuch as the agglutination of bacteria is, in a large measure determined by the magnitude of the potential difference between them and their menstruum it appears that the increases in viscosity of bacterial suspensions which follow upon their agglutination are probably related to or, possibly, are even referable to the effect of pH upon the potential. If it be considered that the potential takes its origin primarily in ion equilibria of the type described by Donnan, it follows that the viscosity of bacterial suspensions and perhaps colloidal suspensions in general are functions of the Donnan equilibria in so far as these equilibria function to affect the apparent volume of the dispersed phase. Further it appears to be unnecessary, as Loeb has done, to postulate the production of sub-microscopic particles to account for increases in viscosity caused by suitable alterations of hydrogen ion concention. These effects of $[H^+]$ can be more simply—and, it appears to us more reasonably explained as consequences of the influences of $[H^+]$ or $[OH^-]$ in causing agglomeration of particles and consequent apparent increase in the volume occupied by the dispersed material.

IV. SUMMARY AND CONCLUSIONS

The general nature of the findings from the experiments with *Bacterium coli* reported here lead to the following conclusions:

1. The viscosity, like certain other properties, of bacterial suspensions appears to be determined by the colloidal nature of bacteria and can be treated as though the bacteria were inert material in the colloidal state.

2. The viscosity of bacterial suspensions is affected by hydrogen ion concentration. Marked increases in viscosity are produced in zones of pH about the two isoelectric points ($pH = 3$ to 4; $pH = 13.0$ to 13.5) where the bacteria show marked acid and alkali agglutination. The effect of hydrogen ion concentration is slight in other pH zones.

3. The viscosity of bacterial suspensions is a function of concentration and is higher the greater the number of bacteria per cubic centimeter of suspension.

4. The relation of bacterial concentration to viscosity is in accord with the general equation of Arrhenius: $\log \eta - \log \eta_0 = \vartheta \phi$, which describes the viscosity of colloidal suspensions as a logarithmic function of the relative volume occupied by the dispersed particles (ϕ).

5. Our findings with bacteria differ from those of Loeb with gelatin and other proteins in that viscosity is *maximal* instead of *minimal* at the isoelectric points.

6. We are led to the conclusion that the increased viscosity which becomes manifest at the isoelectric points of the bacteria is due to an apparent increase in the volume of the dispersed phase which is incidental to the agglutination of the bacteria and the occlusion of water in the clumps of agglutinated particles.

7. From observations made under the microscope it appears that the water occluded in a clump of bacteria behaves as though it were an integral part of the clump. The gross volume of the clump is much greater (60 to 90 times) than the actual volume of the bacteria in the clump. Hence, it follows that even if only a small proportion of the bacteria in a suspension are agglutinated the apparent increase in the volume of the dispersed phase is sufficient to account for the increase in viscosity.

8. The influences of hydrogen ion concentration upon viscosity appear to operate through the mechanism by means of which this ion affects the degree of dispersion or agglutination of bacteria.

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A NEW APPARATUS FOR INTERMITTENT OBSERVATIONS OF PHYSIOLOGICAL CHANGES IN CULTURES OF MICROÖRGANISMS

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In the study of physiological changes in cultures of bacteria, yeasts or other microörganisms, it is of great importance to note the development of various phenomena, by investigating the solutions intermittently. Such intermittent observations of physical and chemical changes in the culture-media, of increase and decrease of the number of bacteria, of the consumption of nutritive substances, of the formation of new substances such as alkali and acids, of changes in hydrogen-ion concentration, etc., are very widely carried out in recent years especially in bacterial chemistry.

Hitherto, two methods had been generally adopted for such investigations. In one method the culture medium is divided into equal volumes and each portion placed in a test-tube, Erlenmeyer flask, or Florence flask. An equal number of microorganisms, which have been cultivated under the same conditions, is inoculated into each portion. All vessels are incubated alike, the same physiological changes occurring in each of them, and the cultures are examined one after another, so that we may know the gradual changes that should take place in any one of them. In the other method a large quantity of the culture is provided in one vessel, and its changes are observed successively by removing a portion with a sterile pipette.

In the first method we can observe the correct results under constant conditions, if the same number of organisms are inoculated into each vessel, and if every vessel is placed in the same conditions, and the same changes occur in all vessels with the same velocity. But these conditions can not always be attained with ease, and it is often the case that various vessels do not show the same stage of change at a given time. Therefore this method can not be relied on except when the experiment needs no great accuracy or when the growth of the microörganisms is very vigorous and not affected by differences in various circumstances.

In the second method the volume of the solution will become smaller and smaller, as the portions are taken from it one by one. For this reason the ratio of the surface of culture-medium to its volume does not remain constant all the time during the experiment, i.e., the air supply for the medium varies as the observation goes further. For example, suppose that we use for the second method a flask with round bottom, whose body has the shape of an exact sphere. Place the center of the sphere at the origin of coördinate axes (fig. 1, *a*). The distance from the center of sphere to the surface of the liquid being p, and the

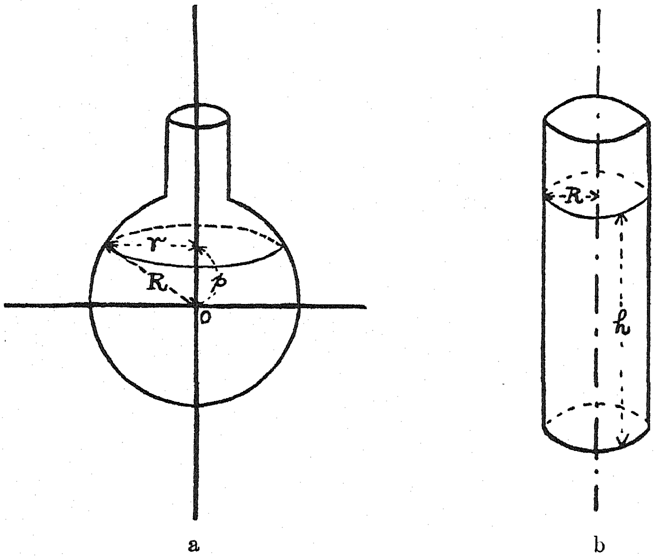

FIG. 1

radius of the sphere being R, the quotient, $\frac{\text{surface}}{\text{volume}}$, varies very irregularly as p varies, that is, the volume of liquid decreases. If we use a large test-tube for the same purpose, imagine the tube as a cylinder, the bottom having the radius R and the length being h, (fig. 1, *b*), the quotient, $\frac{\text{surface}}{\text{volume}}$, increases proportionally as the surface goes down.

Now let us call the ratio of the surface to the volume of the liquid "the surface-coefficient" (or "the surface-volume quotient"). In both of the above examples this coefficient is not constant during the experiment, so when the life-phenomena of the observed microörganisms is appreciably affected by the air-supply the results will be atypical.

The new vessel invented by the author is so constructed, that the quotient, $\frac{\text{surface}}{\text{volume}}$ is not altered by taking off the portions of the liquid in it. In figure 2, a curve represented by the formula

$$y = f\ (x)$$

is rotated around the X-axis, so that a body of revolution will be formed by it. This body is cut with a plane which is vertical to the X-axis, through a point, x, y. Then we have the equation

$$y = Ae^{\frac{kx}{2}}$$

where e is the base of the natural logarithm, and A is a constant, k being the surface coefficient. This formula represents the curve to be obtained.

Since this curve is represented by an exponential function it never intersects the X-axis. For this reason the body of revolution given by it does not satisfy the conditions except when calculated from $-\infty$. But since, in practice, it is impossible to extend the vessel to infinite length, we must either extend the curve sufficiently to approach the X-axis, or cut the body of revolution at some place, which is allowable for the experiment, and substitute the remaining volume in some proper shape.

When, in figure 2, we cut the body at the Y-axis, the total volume of the part which exists on its left hand may be substi-

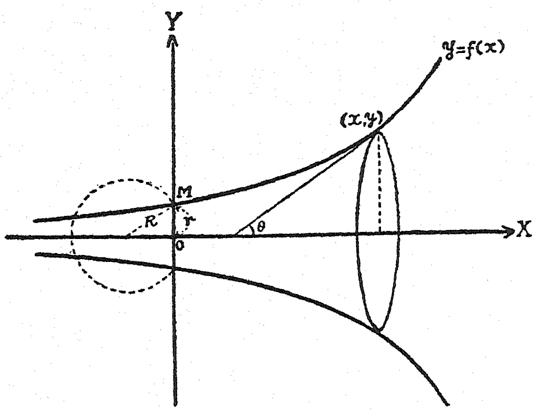

Fig. 2

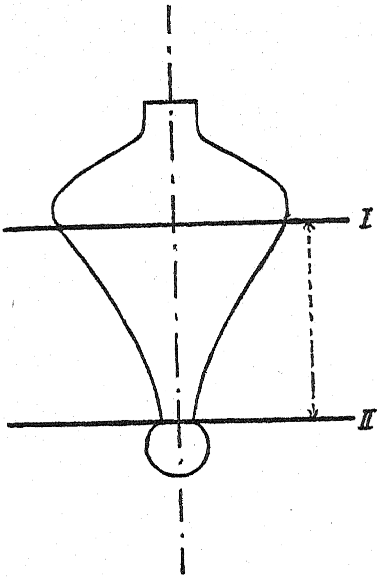

Fig. 3

tuted with a spherical body having the radius R, which can easily be calculated from the following formula.

$$12\,kR^2 - 3\,k^2r^2R^2 - k^2r^4 - 9\,r^2 = 0$$

The bottle which is constructed according to such a principle

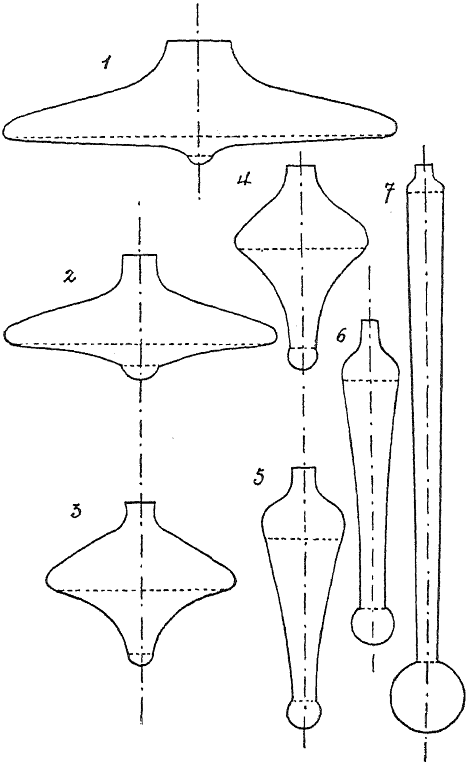

FIG. 4

(1) Surface-coefficient: $k = 5$, can be used between 100 cc.—0.22 cc.
(2) Surface-coefficient: $k = 2$, can be used between 100 cc.—1.7 cc.
(3) Surface-coefficient: $k = 1$, can be used between 100 cc.— 1 cc.
(4) Surface-coefficient: $k = \frac{1}{2}$, can be used between 100 cc.—1.8 cc.
(5) Surface-coefficient: $k = \frac{1}{5}$, can be used between 100 cc.—5.5 cc.
(6) Surface-coefficient: $k = \frac{1}{10}$, can be used between 100 cc.—11 cc.
(7) Surface-coefficient: $k = \frac{1}{50}$, can be used between 100 cc.—50 cc.
The maximum capacity of each being 100 cc.

has the form shown in figure 3. The range in which $\frac{\text{surface}}{\text{volume}} = k$ is satisfied is only the part that is surrounded by the curve

$$y = Ae^{\frac{kx}{2}}$$

namely, the part which is included between the lines I and II in figure 3. In practice it is unusual that all of the solution must be tested. Generally some portion of it will remain at the end of the experiment. If we take this fact into consideration and make R proportionately great, the shape of the bottle will be remarkably simple.

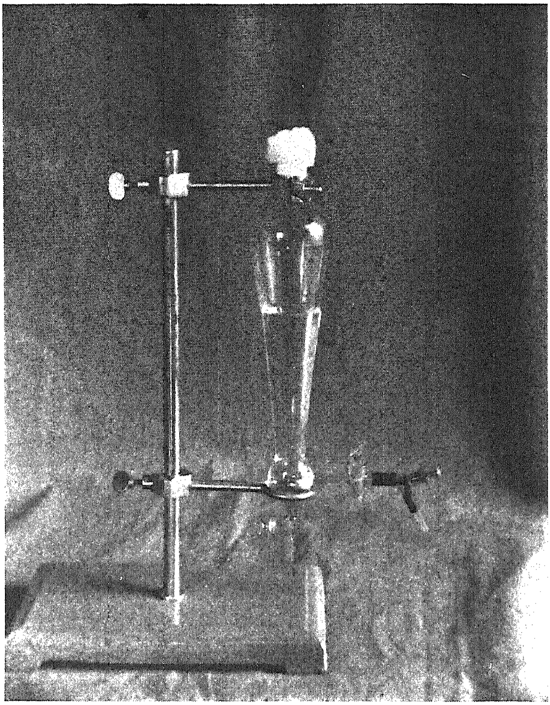

Fig. 5

Figure 4 shows some examples of the bottles, which have different values for k, but the same V. Any type that is appropriate for the experiment can be selected. By the use

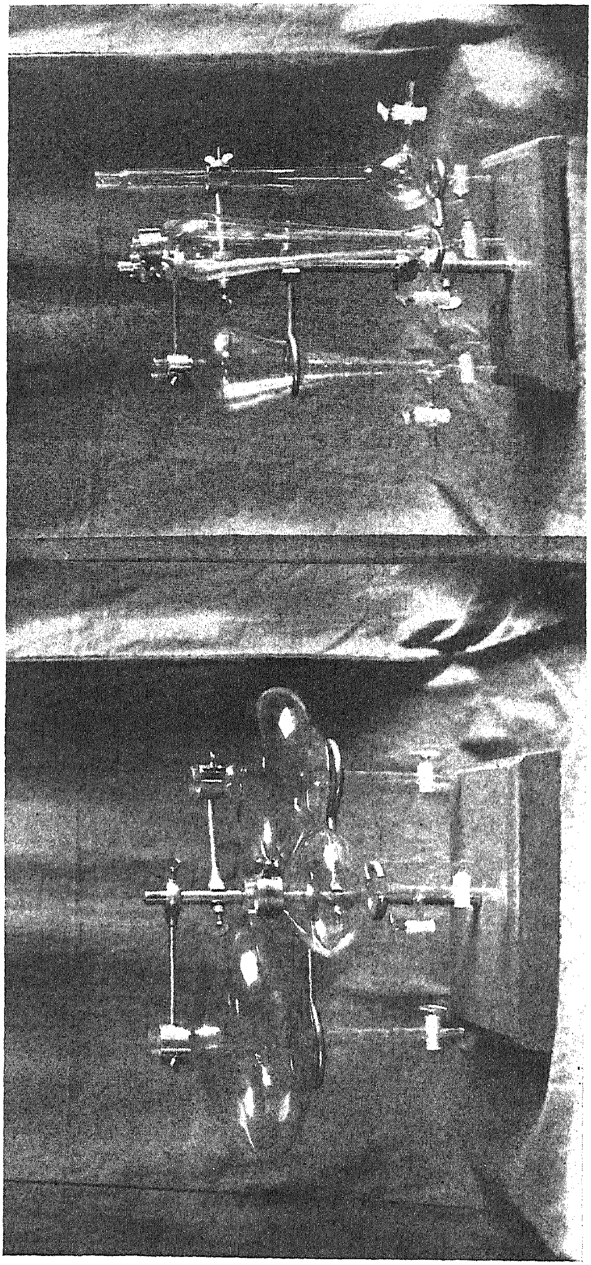

FIG. 6

of the set it is possible to investigate the maximum, optimum and minimum "surface-coefficient" for the growth of micro-organisms.

The bottle which is constructed for the purpose described has two tubes attached to the spherical part, one horizontal and the other vertical, both with glass stopcocks. To the horizontal tube is attached a short rubber tubing with a pinch-cock and a small glass out-let (fig. 5); the solution for test should be taken out by this tubing. The vertical one should not be used during the experiment, but serves for washing out the precipitates or the residuum of the solution at the end of the experiment. The upper opening is closed with the usual cotton plug. The wall of the bottle is graduated to indicate the volume of the contents. The bottles are held by a holder as shown in figure 5. Figure 6 shows a set of the bottles, the maximum V of each being 250 cc., while k is respectively, 2, 1, $\frac{1}{2}$, $\frac{1}{5}$, $\frac{1}{10}$ and $\frac{1}{20}$. The whole set can be placed in the usual thermostat.

In the use of this apparatus, it must not be forgotten that before a portion of the solution is taken out, the bottle must be shaken thoroughly in order to make the solution homogeneous, because it is important to take out numbers of the microörganisms which are representative of the numbers present in the solution as a whole. By the use of this bottle the irregular variation of the air-supply, which had been, hitherto, carelessly overlooked, will be corrected, and at least in this respect our experiments will be rectified.

The author hopes that this bottle may be of importance not only in the physiological study of bacteria and other micro-organisms, but also in other branches of physiological or physical chemistry, for example, in the study of the gas-liquid or liquid-liquid junction.

The experimental study using this bottle in bacteriology is now being carried on by the author and will be reported later.

NOTE ON THE OCCURRENCE OF BACTERIA IN OIL-WELL BRINE SAMPLES

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Last year, in coöperation with the Illinois State Geological Survey, in a study of the oil resources of the state, this laboratory commenced the analysis of a large number of salt brines collected by the geologists during the summer field work. These samples varied in sodium chloride content from less than 0.1 per cent to nearly 10 per cent, and while awaiting analysis in the laboratory some showed a relatively rapid deposition of calcium carbonate. The abundance and persistence of this precipitation could not be attributed to simple loss of free carbon dioxide. In view of the observations by Drew and Vaughn[1] on *Pseudomonas calcis* as an active denitrifier leading to the deposition of calcium carbonate on the Florida coast, our samples were examined for similar organisms.

Peptone-nitrate broths were prepared, containing in one case 3 per cent NaCl and in another 5 per cent NaCl, as follows: 30 or 50 grams NaCl, 2 grams KNO_3, 10 grams peptone, 10 grams KH_2PO_4, 1000 cc. water. One cubic centimeter portions of the samples under investigation were inoculated into sterile tubes of this broth, and denitrification determined by noting nitrite and ammonia production after twenty-four, forty-eight, and ninety-six hours at room temperature.

In a total of 94 samples examined, 12 (or 13 per cent) showed denitrification in a 5 per cent NaCl medium and 31 (or 33 per cent) showed it in a 3 per cent NaCl medium. In the latter medium, 22 others showed abundant growth of non-denitrifying organisms whose physiological characteristics were not investigated. No correlation has yet been found between the occurrence of organisms and the analysis or source of the water. The samples are, however, relatively high in nitrates and in ammonium salts, and the denitrification results in precipitation of calcium carbonate according to the following equations:

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[1] Jour. Wash. Acad., 4, 400–2.

$$Ca(HCO_3)_2 + 2NH_4OH \rightleftharpoons CaCO_3 + (NH_4)_2CO_3 + 2H_2O$$
$$Ca(HCO_3)_2 + (NH_4)_2CO_3 \rightleftharpoons CaCO_3 + 2NH_4HCO_3$$

A series of sub-cultures from the positive tubes showed in all cases the presence of two morphological forms, the one a large stout rod and the other a small slender rod. No attempt was made to obtain their index numbers. However, in view of the predominance of the one only during maximum nitrite production and of the other only during maximum ammonia formation, the presumptive evidence suggests that these two organisms are nitrifying symbionts.

Five sub-cultures, chosen at random, showed the following ammonia productions in the 3 per cent NaCl broth in forty-eight hours at room temperature:

CULTURE NUMBER	MILLIGRAMS OF NH_3 PER LITRE OF BROTH
18	218
54	177
56	170
61	313
97	469

Since the presence of bacteria in oil-well waters was not even suspected at the time of their collection, no attempt was made to exclude possible contamination by soil forms. The work reported in this paper, therefore, does not prove more than the ability of such organisms to live in such waters and to produce marked changes in their composition. The preliminary observations are given here both as a matter of record and as a warning to other workers in the field to observe and to guard against the effects of organisms in such water samples. Further work may well show, however, that bacteria play a significant part in the chemistry, and perhaps the geology, of oil deposits.

SPIROCHAETES IN BALTIMORE MARKET OYSTERS[1]

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Received for publication October 16, 1925

INTRODUCTION

Several investigators in different parts of the world have reported the presence of large, coarse spirochaetes living in the digestive tract of oysters and other shell fish. Certes, Laveran, Mesnil, and Perrin named these spirochaetes *Trypanosoma Balbianii*, while Keysselitz, Fantham and Schellack named them *Spirochaeta Balbianii.* Gross has proposed the generic term *Cristispira* for the large spirochaetes found in the digestive tract of the lamellibranchs.

The present investigation was undertaken, in the first place, to discover whether spirochaetes are found in market oysters in Baltimore; secondly to determine what percentage of the market oysters are infected with spirochaetes; in the third place, to determine the species of spirochaetes found.

REVIEW OF LITERATURE

Certes (1882) first discovered and described a spiral organism which he found in the stomach of the oyster. He believed that this organism was a trypanosome therefore he named it *Trypanosoma Balbianii*, in honor of his teacher and colleague in the College de France.

Laveran and Mesnil (1901) repeated Certes' work and described *Trypanosoma Balbianii* as resembling the spirilla in form and movements, but as differing from the spirilla in the width of the cell body and in the possession of an undulating membrane.

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[1] Thesis presented in partial fulfilment of the requirements for the degree of Doctor of Science in Hygiene in the Department of Bacteriology in the School of Hygiene and Public Health, the Johns Hopkins University, June, 1925.

They concluded that *Trypanosoma Balbianii* is more closely related to the bacteria than to the trypanosomes.

Perrin (1906) found *Trypanosoma Balbianii* in the crystalline style of almost every oyster which he took from the sea and examined immediately.

Keysselitz (1906) described a new species, *Spirochaeta anodontae*, which he found in the crystalline style of *Anodonta mutabilis*, a fresh water mussel.

Fantham (1908) found *Spirochaeta* (*Trypanosoma*) *Balbianii* (*Certes*) in the crystalline styles of the oysters which he purchased in the markets of London. He also found these spirochaetes less frequently in the gut, in the stomach, in the caecum, and in the liver bordering on the stomach. He found *Spirochaeta anodontae* in the crystalline style of *Anodonta mutabilis*.

Schellack (1909) found larger spirochaetes in the inner layer of the crystalline style, while smaller spirochaetes were found between the outer and the inner layer of the style. He is of the opinion that many spirochaetes swim out from the style into the gut.

Gross (1910) working in the Zoological Station at Naples, found two new species, *Cristispira pectinis* and *Cristispira interrogationis*, embedded in the walls of the stomach, in the walls of the intestine, and occasionally in the crystalline style of *Pecten Jacobeus* (scallop). In the same year Gross described two new species, *Saprospira grandis* and *Saprospira nana*.

Bosanquet (1910) reported observations on *Spirochaeta anodontae* and concluded that these spiral organisms are bacteria.

Dobell (1910) found a large species of *Cristispira* in the crystalline style of *Venus* (*Meretrix*) *casta Chem.*, the clam, obtained from the salt water lake Tamblegan in the island of Ceylon.

During the summer of 1916 Noguchi studied the spirochaetes found in shell fish in the vicinity of Woods Hole, Massachusetts, and in Long Island Sound. He found the cristispiras most frequently in the styles of oysters, next in clams, and then in the mussels. No other species examined showed any cristispiras.

EXPERIMENTAL

a. Materials and methods

The spirochaetes here described were obtained from oysters purchased in the markets of Baltimore. There were two varieties of oysters. One variety apparently came from muddy oyster beds, and still had fine silt adhering to the shells. The other type was comparatively free from mud and apparently came from sandy or gravelly oyster beds. The muddy oysters were more highly infected with spirochaetes than the clean specimens.

The oysters were opened by inserting an oyster knife between the lips of the shells, cutting the abductor muscle, removing the right shell, and draining off the liquor. Care was taken to avoid cutting into the body of the oyster. The gills were pulled dorsally in order to expose the intestine, the caecum, and a portion of the liver.

During the first observations the intestine was split longitudinally in order to locate the crystalline style lying in the lumen of the gut near the liver. Fragments of the crystalline style were found only in one oyster, because the market oysters are usually not fresh, and the crystalline style disappears in two hours after the removal of the oyster from its natural habitat. Since no crystalline styles were found, specimens for microscopic examination were taken from the stomach and from the intestine by inserting the point of a Pasteur pipette through the intestinal wall into the lumen and sucking up some of the liquid.

A hanging drop preparation was made and examined microscopically in order to determine whether spirochaetes were present in the oyster before making stained preparations. If no spirochaetes were found in the intestine, the contents of the stomach were examined for spirochaetes in the same way. Often the spirochaetes were not found in the intestine, while they were found in the contents of the stomach. This was especially the case with oysters that had been kept in the ice box for several days. It appeared that the spirochaetes migrate towards the stomach as the oyster slowly dries up (table 1). In other cases the spirochaetes were just as numerous in the intestine as they were in the stomach after storage in the ice box for seventeen days.

There was no way to determine how long the oysters had been out of water before they were brought into the laboratory. It is safe to assume, however, that they had been out of water at least one week when purchased in the market. They were then stored in the ice box from one to seventeen days before they were examined for spirochaetes. Table 1 shows that living spirochaetes were observed in the digestive tract of the oyster during this whole period.

TABLE 1

Table showing the infection of oysters by spirochaetes

NUMBER OF DAYS OYSTERS WERE KEPT IN ICE BOX BEFORE EXAMINED	NUMBER OF OYSTERS EXAMINED	NUMBER OF OYSTERS		SPIROCHAETES FOUND IN	
		Negative	Positive	Intestine	Stomach
0	17	3	14	13	14
2	5	1	4	1	4
2	4	0	4	4	4
3	17	2	15	13	15
4	6	0	6	5	6
5	3	1	2	2	2
6	7	0	7	7	7
7	1	0	1	1	1
8	2	0	2	2	2
9	9	0	9	9	9
12	6	0	6	6	6
15	1	0	1	1	1
16	1	0	1	1	1
17	2	0	2	2	2
Totals........	81	7	74	67	74
Percentage...	100	9	91	83	91

Analysis of table 1 shows that out of 81 oysters examined only 7, or 9 per cent, did not contain spirochaetes. The table further shows that the spirochaetes were found more frequently in the stomach (91 per cent) than in the intestine (83 per cent). It is also evident that the oysters which were kept in the ice box for seventeen days showed sporochaetes both in the stomach and in the intestine. As long as the oyster retains some of its liquid in the digestive tract the spirochaetes are apparently able to survive. These spirochaetes have no pathological significance for man whatsoever. They simply are the normal inhabitants of the shellfish.

The ability of the spirochaetes to live in the drying oyster was further tested by opening the oyster and removing the right shell. The gills were pulled back, exposing the digestive tract. The presence of spirochaetes was determined by microscopic examination of hanging drop preparations. If the oyster contained spirochaetes it was put into an open Petri dish and set in the ice box. Daily microscopic examinations were made as long as the spirochaetes were motile and the results are shown in table 2.

TABLE 2

Record of spirochaetes found in the digestive tract of oysters after being open for several days

EXPERIMENT	SPIROCHAETES FOUND IN THE DIGESTIVE TRACT OF OYSTERS AFTER BEING OPEN FOR							
	One day	Two days	Three days	Four days	Five days	Six days	Seven days	Eight days
I	+	+	+	+	+	+	+	0
II	+	+	+	+	+	+	0	
III	+	+	+	0				
IV	+	0						
V	+	+	+	+	+	0		

+ = spirochaetes found; 0 = spirochaetes not found.

Table 2 shows that the spirochaetes remained alive for some time in the open oyster. In observation I the spirochaetes were found on the seventh day, while in IV they were found only on the first day. The spirochaetes survived as long as there was some fluid in the lumen of the digestive tract. As the intestine began to disintegrate the number of motile spirochaetes decreased. Gradually the gut became a soft, pulpy mass, and with this decomposition the motile spirochaetes disappeared entirely.

The spirochaetes were stained by various methods which are described as follows:

Air-dried films were fixed in absolute alcohol for thirty minutes, washed in water, and stained in Giemsa's solution overnight (figs. 1 to 6, 10, plate 1; fig. 13, plate 2; figs. 20 and 24, plate 3).

Air-dried films were fixed by heat and stained with Giemsa's solution overnight (figs 8, 9 and 15, plate 2).

The moist films were exposed to osmic acid vapor for one minute, fixed in absolute alcohol for thirty minutes, washed in water, covered with Lugol's solution for five minutes, washed in water, flooded with 0.5 per cent sodium thiosulphate for five minutes, washed in water, and stained in Giemsa's solution overnight (fig. 18, plate 2; figs. 21, 23, 25 and 26, plate 3; figs. 27, 32, 33 and 37, plate 4).

The moist (wet) films were exposed to osmic acid vapor for one minute, fixed in absolute alcohol for thirty minutes, washed in water, immersed in 5 per cent picric acid for thirty minutes, washed in water, and stained in Giemsa's solution overnight (figs. 7, 10a and 11, plate 1; figs. 14, 16 and 19, plate 2; figs. 28, 29, 31, 34, 35 and 36, plate 4).

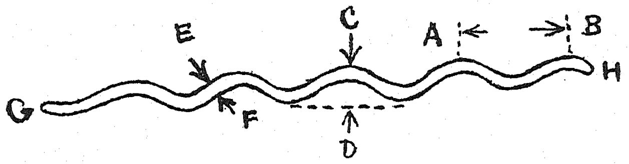
The moist films were exposed to osmic acid vapor for one minute, immersed in 5 per cent picric acid for thirty minutes, allowed to remain in a dish of clean water for ten minutes in order to wash out the picric acid, rinsed thoroughly in fresh water, stained in Giemsa's solution (1 drop of the stain to 1 cc. distilled water and 1 drop of potassium carbonate to each 10 cc. of the stain) for three days. The preparations were then allowed to remain in a dish of clean water for thirty minutes in order to wash out all superfluous stain, rinsed in fresh water, air-dried and examined. This method is most affective in staining the crista although there is a tendency to overstain and thus lose to a certain degree the clearness of the internal structure. Experience with the reagents shows the best technique for good results. With this method the spirochaetes stain dark purple and the crista stains pink (figs. 38 to 58, plates 5 and 6).

The wet films were fixed in Fontana's solution no. 1 (acetic acid, one part; formalin 2 parts; distilled water, 100 parts) for two minutes, washed in water, immersed in absolute alcohol for thirty minutes, washed in water and stained in Giemsa's solution for one hour (fig. 30, plate 4).

The wet film was exposed to formaldehyde gas for five minutes, allowed to dry, then immersed in absolute alcohol for thirty minutes, washed in water, and stained with Giemsa's solution for two hours (figs. 12 and 14, plate 2).

b. Definition of terms

The definition of a Spirochaete according to Swellengrebel is as follows: Spirochaetes are spiral microörganisms in which the individual cell occupies one or more complete spiral turns. The cell body is made up of an axial filament surrounded by periplast or various modifications of this structure. Terminal prolongations of periplast or of axial filament are present in at least one genus. A chambered or honey-combed arrangement is present in two genera. They possess no true bacterial flagella. Volutin granules are present in nearly all forms of spirochaetes and chromidial granules are found in many species. It is probable that the spirochaetes have no true chromatin.[2] The cell bodies are always elastic. The spirochaetes are free living, saprophytic, or parasitic, pathogenic and non-pathogenic microörganisms.



TEXT-FIG. 1. SHOWING METHODS OF MEASURING SPIROCHAETES

The terms used in this paper to describe the measurements of the spirochaetes are those used in the literature dealing with this subject. For the sake of clearness they are stated as follows:

The spiral amplitude (wave length of the spiral) is the length of one complete spiral turn, measured from the apex of one crest to the apex of the next crest as is indicated by *AB* in text-figure 1.

The spiral depth (width of the spiral) is the diameter of the spiral measured transversely on the outside of two opposite apices of the spiral as is indicated by *CD* in text-figure 1.

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[2] The term chromatin as used in this discussion is not limited strictly to the nuclear matter of the cell, but includes the elements in the protoplasm that take the chromatin stain.

The width of the cell body is the transverse distance of the cell body measured perpendicularly to the long axis as is shown by *EF* in text-figure 1.

The length of the cell body is the distance between the two poles as is indicated by *GH* in text-figure 1.

The spirochaetes found in the oysters and described in this paper are classified according to whether they possess or do not possess crista. Those sporichaetes that do not possess crista are grouped under the genus *Saprospira Gross.* This genus is further subdivided into two divisions. One division includes the saprospiras having pointed ends, while the other division includes the saprospiras having rounded ends. All the species of saprospiras are classified under these two divisions, and then identified by their morphological characteristics.

The oyster spirochaetes having crista are classified under the genus *Cristispira Gross.* This genus is further subdivided into two divisions. One division includes the cristispiras having pointed ends, while the second division includes the cristispiras having rounded ends. All species of cristispiras are classified under these two divisions and then identified by their morphological characteristics.

c. Descriptions of species

Four different types of saprospira were found and investigated. One of these species proved to be *Saprospira grandis*, two types of *Saprospira lepta* and one a type of *Saprospira puncta.* Seven species of cristispira, *Cristispira Balbianii*, *Cristispira anodontae*, *Cristispira spiculifera*, *Cristispira modiolae*, *Cristispira modiolae var A* nov. var., *Cristispira mina* nov. spec., *Cristispira tenua* nov. spec. and *Spirillum ostrae* were also found and described.

1. *Saprospira grandis* Gross. *Saprospira grandis* was observed in four stained preparations. It was found in the digestive tract of the oyster less frequently than *Cristispira Balbianii.* It possesses a chambered structure, but no crista was observed on any of the specimens that were studied (figs. 20 to 26, plate 3).

The fully grown *Saprospira grandis* averages about 80 micra in length by 1.2 micron in width, while the extremes are 52 to 90 micra for the length and 1.2 to 1.4 micron for the width. The average spiral amplitude is 24 micra, while the extremes are 20 to 28 micra. The number of turns varies from 3 to 4½. The wide variations in the wave length and in the width of the spiral are due to the different methods of fixing and staining.

The chambered structure found in all specimens examined was the same, irrespective of whether the spirochaetes were stained in dry or in wet films. The chromatin appeared in narrow transverse bars equidistant from each other.

Gross has described the heavily stained areas found at the tips of the poles both in saprospiras and in cristispiras as "Polkappen" or polar caps. These structures were present in nearly all the specimens of *Saprospira grandis* observed. They appeared to be nothing more than chromatin material segregated at the end portions of the organisms.

A heavy border line surrounding the entire cell body was observed in stained preparations. In hanging drop preparations a distinct, shadow-like margin was seen about the cell body of this species. This apparently represents the cell membrane.

These organisms move like a corkscrew rotating through the medium. When the motion slows down, the moving saprospira gives the appearance of a chain made up of a figure 8, due to the spirals rotating on the long axis. Frequently, contracting and stretching motions can be observed, simultanuous with the rotating movements. The forward movement of the saprospira appears to be due to waves of peristalsis-like contractions of the protoplasm or of the elastic cell membrane. According to Fantham these impulses are due to contractile myonemes or muscular fibrils, embedded in the periplast at right angles to the long axis. He has described 4 to 6 successive impulses passing simultaneously over the organism. In this investigation only one impulse was observed at any time. It is possible that the motility is caused by a motile "posterior" end portion. This is suggested by the observations on specimens having a retarded movement.

The method of multiplication was not observed in the living specimens, although one stained preparation (fig. 17, plate 2) shows that multiplication takes place by a kind of transverse division, corresponding to what Gross called "incurvation." This he described as follows: As the cristispira approaches the stage of cell division it bends (incurvates) a short distance from the middle. During this incurvation the two halves beat and lash violently, then the lashing ceases for intervals of one-half of one second. The end of the unbent half glides over the other half until both ends come together, then the two halves intertwine and simulate a stage of longitudinal division as is illustrated by figure 16, plate 2. At this time, constriction of the cell body begins at the point of incurvation and the process of division is completed in thirty to sixty minutes. After apparently complete constriction the new daughter cells do not separate but seem to remain attached, whipping and lashing movements taking place as if the two halves were struggling to detach themselves from each other.

2. *Saprospira lepta* nov. spec. This organism was observed in two stained preparations, taken from two different oysters on two different occasions. Whenever it is seen in stained preparations it is very characteristic because it appears coiled and twisted into knots of various designs. Often one end bends and forms a loop while the remainder of the cell body is straight (figs. 7 and 11, plate 1). The chromatin is segregated in wide blocks, although narrow transverse bars are also found with clear, circular, unstained areas between each block of chromatin material. Slight bulging is evident at the circular, unstained areas and occasionally the ends show bulging if there are unstained portions there. This spirochaete looks like a chain with long narrow solid links connected by larger, circular links. The ends taper down to sharp points, although individuals are found with ends less pointed, and occasionally one end is sharp while the other tapers down to a blunt point.

Accurate measurements of the spiral amplitude and the spiral width are impossible on account of the irregular spirals. The width and the length of the cell body can be determined however. The average spirochaete is 70 micra long by 0.5 micron wide, with extremes of 54 to 92 micra for the length, while the width of the cell body is constant. The average spiral amplitude is 8 micra with extremes of 5 to 13 micra. The average spiral width is 1.6 micron with extremes of 1.6 to 4.8 micra. The average number of turns is 6 with extremes of 3½ to 10 turns. The longest individual observed measured 92 micra.

A crista was never found on this spirochaete, although crista were observed on other species in the same film (fig. 19, plate 2, and fig. 36, plate 4).

This spirochaete was never seen in the living condition so that its manner of motility could not be determined.

The process of division was never observed either in the living or in stained specimens. On the basis of mathematical measurements multiplication probably takes place by transverse division. Spirochaetes 92 micra in length, nearly twice as long as the shortest specimens, 54 micra long, were found, but no specimens were observed that were twice as wide as the narrowest individual. The width of the cell body is constant, i.e., it is 0.5 micron. It is possible that the coiling and twisting are stages of incurvation in the process of transverse division, but no constriction of the cell body were observed on any specimens.

In stained preparations this spirochaete shows what Perrin has described in *Trypanosoma Balbianii* as coiling and encystment. In *Saprospira lepta* the coiling was undoubtedly due to unfavorable conditions during the fixation and the staining of the films. This spirochaete is a type of saprospira, although it differs from *Saprospira grandis* Gross in having pointed ends, in the distribution of the chromatin, and in the irregularity of the spirals. This species has not been described in the literature, therefore the name *Saprospira lepta* (a thin or fine saprospira) is suggested, to distinguish it from *Saprospira grandis* and *Saprospira nana* Gross.

3. *Saprospira lepta var. A* nov. var. This spirochaete (figs. 10 and 10a, plate 1) and *Saprospira lepta* were observed in the same stained preparations. They have many characteristics in common but they differ in the shape of the ends thus necessitating separate descriptions.

Saprospira lepta var. A is found coiled and twisted in knots of various designs, and in a straight position. The spirals are irregular and the crests form corners rather than smooth curves. The cytoplasm coagulates in solid, long blocks, with circular, clear areas between them (fig. 10a, plate 1). Some specimens have the cytoplasm in long and short blocks, in transverse bars, or in narrow transverse lines with the typical clear, circular areas between each block. A slight bulging is noticeable at each unstained area and occasionally the terminal portions show bulging (fig. 10, plate 1). These appearances may have been caused by the heating during the process of staining.

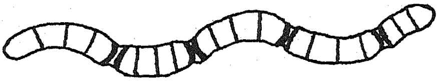
Accurate measurements of these spirochaetes is impossible on account of their irregular spirals. The average length of the cell body is 60 micra, with extremes of 45 to 72 micra. The length of the spiral is 20 micra. The width of the spiral is 4 micra with extremes of 3.2 to 5.2 micra. The number of turns is 2 to 4, averaging about 3 turns for the fully grown individual.

Saprospira nana Gross and *Saprospira lepta var. A* are similar in having thin (narrow) cell bodies, but they differ in all other points. *Saprospira lepta var. A* and *Saprospira lepta* are similar in having the cytoplasm in wide blocks; in having clear circular areas between the dark stained portions; in forming coils and twisted knots; in having thin (narrow) cell bodies. They differ, however, in the shape of the ends, in the wave length and the width of the spiral, and in the number of turns. Since this spirochaete corresponds to neither of the spirochaetes that have been described it is regarded as a new variety and named *Saprospira lepta var. A* nov. var.

4. *Saprospira puncta* nov. spec. *Saprospira puncta* is similar to *Saprospira grandis* except that its ends are pointed and the chromatin is arranged in transverse bars farther apart. It is the longest oyster spirochaete which we found and was present in five different stained preparations. In one of these preparations it was observed in considerable numbers.

The contours of the cell body are parallel except that the ends taper down to sharp points. In some specimens the spirals are regular and deep. In other specimens a portion of the cell body forms regular spirals, while the remainder may be straight. Specimens that have lost their spiral shape are twisted into s-shape, into c-shape, etc. The terminal portions are straight, pointing in the direction of the long axis.

The chromatin material (dark stained areas) segregates either in narrow transverse bars or in wide blocks, which are farther apart than the transverse bars in *Saprospira grandis* or in cristispiras. Chromatin is found in the terminal points, thus giving them the appearance of the head of a javelin. Constrictions in the cell membrane were observed wherever the chromatin formed wide blocks or bands. Figure 12, plate 2, shows such a constriction near the middle. It certainly suggests simple transverse division. Figure 13 shows constrictions at three points, while Figure 14 shows four such constictions. Figure 15 shows five constrictions and the chromatin between them appears porous, due to the plasmolized condition of the spirochaete, as is indicated by the wide portions between the constricted points. It is possible that these numerous constrictions indicate multiple transverse fission.



TEXT-FIG. 1a. SHOWING PAIRS OF THICK TRANSVERSE BARS AT SHORT INTERVALS

The transverse bars in one specimen, 86 micra long and 1 micron wide, shows an unusual arrangement. At short intervals a pair of transverse, crescent-shape, thick bars is found close to each other, while two or three bars are present in the cytoplasm between each set of these double bars (text-fig. 1a). A constriction of the cell membrane can be seen opposite each pair of double bars. This appearance is present only in the middle portion of the cell body, but the regular transverse bars are closer together towards the extremities than they are in the middle portion. This arrangement is similar to the multiple transverse fission described by Gross in *Saprospira grandis.* It also resembles the constrictions of the cell body described in the preceding paragraph.

The presence of chromatin spirals in the cytoplasm of the oyster spirochaetes has been observed on several occasions but never with any degree of clearness. In this specimen zigzag lines were observed running through the cytoplasm. One line was wider and approximately at right angles to the main axis, the other line was narrower and diagonal to the main axis. Chromatin granules were observed at the junction of these lines and the cell membrane. This structure was best observed in spirochaetes having lightly stained portions against a back ground free from debris. It is possible that these zigzag lines are part of the normal structure of oyster spirochaetes but they cannot be demonstrated by the present methods of staining with sufficient clearness to justify any conclusion.

The measurements were made on specimens having comparatively normal spirals. The longest undivided spirochaete is 100 micra long. The average *Saprospira puncta* is 80 micra long with extremes of 60 to 100 micra. The width of the cell body is 1 micron with extremes of 0.9 to 1.2 micron. The spiral amplitude is 6 micra with extremes of 4 to 8 micra. The average number of turns is 3 with extremes of 2½ to 6 turns.

The process of multiplication was never observed in the living spirochaetes. Stained specimens showed typical incurvation which suggests that multiplication takes place by transverse division. No specimens were found that were twice as wide as the narrowest spirochaete. The widest saprospira observed was 1.2 micron and the narrowest 0.9 micron. On the other hand organisms nearly twice as long as the shortest were numerous. The longest spirochaete observed measured 100 micra and the shortest 60 micra.

The width and the wave length of the spirals in this species are similar to those of the larger type of *Saprospiras* (figs. 12 and 13, plate 2), but the transverse bars are farther apart and the ends are pointed. Morphologically this spirochaete is similar to Schellack's *Spirochaeta tapetos* with the exception of the pointed ends and the absence of the crista. This spirochaete resembles Keysselitz's *Cristispira anodontae* except that it has no crista. It also resembles Schellack's *Spirochaeta ostrae* (a cristispira) in the shape of the ends, but it has no crista and the spiral amplitude is very long. It seems clear that this spirochaete belongs to the genus *Saprospira Gross*. The name *Saprospira puncta*, (puncta = pointed) is suggested as descriptive of its characteristics.

5. *Cristispira Balbianii* (Certes) Gross. This species of cristispira was most common in the specimens taken from the intestinal tract of the oyster. Figures 16, and 19, plate 2; figures 27, 33, 36, and 37, plate 4; figures 40 to 43 and 46, plate 5; figures 51 to 55, plate 6, are types of *Cristispira Balbianii*, although they show differences which were caused by the methods of fixation and staining.

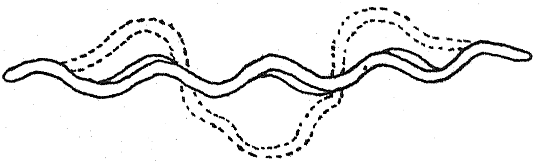
The average *Cristispira Balbianii* measures 60 micra inlength and 0.8 micron in width, with extremes of 39 to 78 micra for the length and 0.8 to 1 micron for the width. The average spiral amplitude is 8 micra, with extremes of 6 to 8 micra. The average width of the spiral is 1.6 micron with extremes of 1.2 to 2.4 micra. Slight variations in dimensions were observed and probably can be traced to different methods of staining.

The cell body of *Cristispira Balbianii* is spiral, flexuous, with a crista running obliquely to the main axis. The average number of complete turns is 7, with extremes of 4½ to 11 turns. The contours of the cell body are parallel , although the ends are rounded.

The chambered structure of *Cristispira Balbianii* is most evident in stained preparations, although in some specimens the chromatin is segregated along the cell membrane, as is shown in figure 18, plate 2; figure 36, plate 4. The chromatin is also arranged in wide transverse bars, as is shown in figures 28, 32 and 37, plate 4. In several specimens it was present in a series of broken lines running longitudinally through the middle of the cytoplasm. Specimens that stained solidly were also observed (fig. 16, plate, 2; figs. 31 and 33, plate 4).

These cristispiras move so rapidly that they cannot be seen easily under the low power objective, but their presence is detected by the disturbance of the medium caused by their motion. They move across the microscopic field with lightning speed, dashing in one direction and suddenly coming to a dead stop, then immediately darting in the opposite direction. Their spiral form can be observed during this momentary stop. Careful observations on their manner of motility are not possible, except in specimens with retarded movement due to exhaustion and to the drying of the medium. As the motility becomes retarded the cristispiras show a serpentine twisting. In addition to their regular spirals the cristispiras form a secondary spiral of wider and greater amplitude as is indicated by the dotted lines in text-figure 2. The secondary spiral disappears and the motility of the cristispiras gradually ceases. At this time the cristispiras begin to vibrate apparently from the peristalsis-like contractions of the elastic cell body. During the vibrating the normal spiral is distorted and appears irregular. When the cristispiras cease to move they take the true spiral position as shown in plate 4, which approximates the position of the living organism.

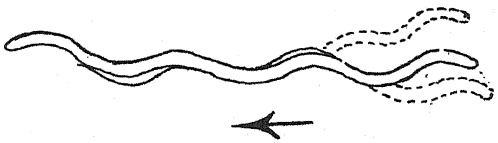
The cristispiras move with equal velocity in either direction and have no anterior-posterior polarity. They show rotation on their long axis, boring through the medium like a cork screw. During the normal spiral movement the first spiral follows in the path of the one end which may be called the "anterior pole," the second spiral follows in the path of the first spiral, the third spiral follows the second spiral, etc. In other instances the normal spiral follows the path of the secondary spiral and irregular bendings and twistings of the organism are frequent. The direction of motion is reversed by a reversal of the direction of the spiral rotation. In other words the movement in one direction is accomplished with the spirals rotating clockwise, while the movement in the opposite direction is accomplished with the spirals rotating the other way.



TEXT-FIG. 2

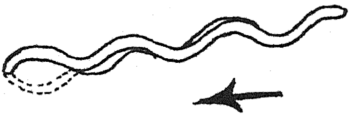
The dotted lines indicate the position of the secondary spiral. The solid lines indicate the normal spiral.

The cristispiras also show a rotating and lashing movement when they meet resistance or become fixed in the drying margin of the film. The spiral movement begins from the fixed end and is transmitted through the entire organism. The spirochaete rotates on its long axis and at the same time lashing movements of the "posterior" third of the cell body are seen as is shown by the dotted lines in text-figure 3. Similar rotating and lashing movements are observed in freely moving cristispiras. One-third of the "posterior" cell body flaps like a fish tail, while the second half of the last "posterior" turn appears slightly "hooked" giving the appearance of an ellipse as the cristispira rotates on its long axis (text-fig. 4). The rotating "posterior" spiral resembles the appearance of the "hooked" ends of a rapidly moving leptospira. When the direction of motion is reversed the "posterior" portion does the flapping while the "anterior" becomes quiescent. The lashing "posterior" ends of the cristispira are similar to what Noguchi (1918) calls "motile end portions" in spirochaetes. It is possible that the forward movement of the cristispira is caused by the rotating and the flapping movements of the "posterior" motile end portions of the cell body. This flapping is similar in execution and in effect to the lateral flappings of a fish tail which forces the fish forward. It further resembles the tail movements of a moving eel or of a swimming water snake.



TEXT-FIG. 3

The dotted lines indicate the rotating-lashing movements of the cristispira. The arrow indicates the direction of motion.

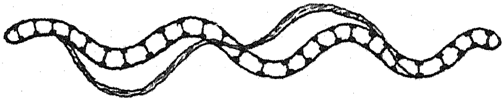


TEXT-FIG. 4

The arrow shows the direction of the motion. The dotted lines indicate the path of the "hooked" end portion.

Vital staining. A hanging drop preparation was made from the liquid in the intestine or in the stomach and was examined microscopically. If cristispiras were present a small amount (equal to half the volume of the hanging drop) of 1 per cent aqueous solution of the dye was mixed with the hanging drop directly on the same cover glass. This was then inverted, placed on a clean glass slide, and examined under the oil immersion objective. It was necessary to make the film very thin because it was impossible to focus accurately on the spirochaetes through a thick film. The following dyes were used: melachite green, safranin, Bismark brown, and neutral red.

A 0.3 per cent aqueous solution of melachite green does not stain the cristispiras, while 1 per cent aqueous solution stains them uniformly green. The cell membrane stains darker green than the cytoplasm. The transverse septa (bars) are very distinct, although five minutes passed before they took the stain. The septa are very delicate, transverse partitions distributed equidistantly in the cytoplasm as is shown in text-figure 5. Chromatin granules can be observed at the junction of the septa and the cell membrane. Unfortunately the spirochaetes die immediately upon coming in contact with the dye and exhaustive study of the cytoplasm is not possible. The outer margin of the crista stains green while the area between this margin and the cell membrane does not take the stain. The complete course of the crista could not be followed because it was not stained sufficiently.



TEXT-FIG. 5. TRANSVERSE SEPTA AS THEY ARE SHOWN BY INTRA VITAM METHOD OF STAINING

A 1 per cent aqueous solution of safranin stains the cristispiras lightly at first but the staining becomes more intense in the course of fifteen minutes. The cell membrane (dark border line) stains darker than the cytoplasm, while the transverse septa appear as thin lines. No granules at the junction of the septa and the cell membrane are visible at first but they appear after ten to fifteen minutes of staining (text-fig. 5). The crista appears narrow, i.e., no wide portions are visible, and its course can be accurately traced. The outer margin stains moderately red, while the area between it and the cell membrane does not stain. The cristispiras die as they come in contact with the stain so that the staining reaction of the living organism could not be observed.

A 1 per cent aqueous solution of Bismarck brown stains the cristispiras evenly, giving them a delicate brown tinge. The transverse septa and the chromatin granules are not as pronounced as they are with melachite green, safranin, or neutral red. The outer margin of the crista is marked, as a light brown line running diagonally to the main axis of the cristispira.

A 1 per cent aqueous solution of neutral red stains the cristispira evenly red, but they die immediately upon coming in contact with the stain. The transverse septa are delicate but darker red than the cytoplasm, while the cell membrane stains dark red. The granules at the junction of the transverse septa and cell membrane are very marked and stain dark red. The crista is wider and its outer margin stains heavily, while the space between this margin and the cell membrane does not take the dye. The crista stains deeply so that its course can easily be traced.

The crista. The crista is a keel-like ridge running spirally on the outside of the organism, making one complete spiral turn to two complete turns of the cell body. The outer margin of the crista is thicker and can be observed in the living organism, while the margin attached to the cell body is thin and cannot be observed clearly. The crista merges with the cell membrane before it reaches the poles. In good preparations the crista shows a fibrillar structure (figs. 27, and 28, plate 4). Observations on the motility of these forms suggest that the crista serves as a rudder to impart the spiral movement to the cristispira. No independent movement of the crista can be observed in the living specimens. The crista stains light pink with Giemsa's solution as contrasted to the purple cell body.

Further observations on the crista revealed a change similar to plasmolysis. In a preparation exposed to osmic acid vapor for one

Vital staining. A hanging drop preparation was made from the liquid in the intestine or in the stomach and was examined microscopically. If cristispiras were present a small amount (equal to half the volume of the hanging drop) of 1 per cent aqueous solution of the dye was mixed with the hanging drop directly on the same cover glass. This was then inverted, placed on a clean glass slide, and examined under the oil immersion objective. It was necessary to make the film very thin because it was impossible to focus accurately on the spirochaetes through a thick film. The following dyes were used: melachite green, safranin, Bismark brown, and neutral red.

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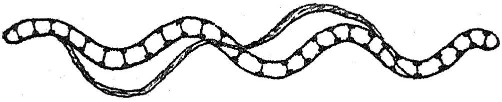

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A 1 per cent aqueous solution of neutral red stains the cristispira evenly red, but they die immediately upon coming in contact with the stain. The transverse septa are delicate but darker red than the cytoplasm, while the cell membrane stains dark red. The granules at the junction of the transverse septa and cell membrane are very marked and stain dark red. The crista is wider and its outer margin stains heavily, while the space between this margin and the cell membrane does not take the dye. The crista stains deeply so that its course can easily be traced.

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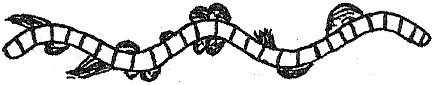
Further observations on the crista revealed a change similar to plasmolysis. In a preparation exposed to osmic acid vapor for one minute and put directly into Giemsa's solution the crista appeared much wider, as if pulled away from the cell body; otherwise the cristispiras appeared normal. In some specimens the crista was frayed out and appeared fibrillar. Stained preparations fixed in picric acid showed many cristispiras whose cristas appeared like a series of semicircles adhering to the side of the cell body. At several points the crista showed a ragged appearance indicating that it had been torn from the cell body (text-fig. 6). The margins of these semicircles were stained dark red, while the enclosed areas stained a light pink.

Figures 16, 18 and 19, plate 2 and figures 51, 53 and 54, plate 6, illustrate the process of incurvation during transverse division. Figure 51 shows that transverse division does not always take place at the middle of the spirochaete. One daughter cell is 42 micra long while the other is 48 micra long. Figure 44, plate 5, represents a young individual of *Cristispira Balbianii* 39 micra long, while figure 41 represents a fully grown individual, 75 micra long, before a transverse division.



TEXT-FIG. 6. THE SHADED AREAS INDICATE THE PLASMOLYSIS OF THE CRISTA

Certes described *Trypanosoma Balbianii* as 100 to 120 micra long by 3 to 5 micra wide with slightly tapering terminal portions, and bluntly rounded ends. Perrin described it as from 26 to 100 micra long and 5.3 micra wide. The cell body was parallel throughout, possessed 3 to 8 spiral turns and the ends were rounded. Fantham described *Spirochaeta Balbianii* as a long, sinuous, thread-like organism 50 to 150 micra long by 2 to 3 micra wide, with rounded ends. Schellack described it as averaging 39 micra long by 1.3 micron wide with extremes of 35 to 42 micra for the length and 1.1 to 1.5 micron for the width, with rounded ends, but no terminal appendages. Noguchi described *Cristispira Balbianii* as "a long, flexible cylinder, with blunt extremities towards which the diameter gradually diminishes." Gross described *Cristispira pectinis* as 72 micra long by 1.5 micron wide, the width of the spiral 4 micra, the terminal portions tapering slightly and the ends bluntly rounded without terminal filaments. Dobell described *Cristispira veneris* as averaging 50 to 60 micra long by 1.3 micron wide with extremes of 1 to 1.9 micron for the width. The terminal portions taper slightly, the ends are blunt without polar caps and without terminal filaments. The *Cristispira Balbianii*, described in this paper, does not correspond to any of the descriptions given by other investigators in respect to the width and the length of the cell body which is described as 1.1 to 5.3 micra wide by 26 to 150 micra long. In other respects the description and the drawings of the spirochaete presented in this paper coincide with the descriptions, drawings, and microphotographs of *Cristispira Balbianii* as presented by each previous investigator. On the basis of similarity in descriptions and drawings the spirochaete described in this investigation is identified as *Cristispira Balbianii*.

6. *Cristispira anodontae* Keysselitz. This organism (figs. 34 and 35, plate 4; figs. 38 and 39, plate 5) was found but a few times in the gut of the oyster. The average fully grown individual measures 70 micra in length with extremes of 44 to 88 micra. The average spirochaete measures 0.8 micron wide with extremes of 0.8 to 1.2 micron. The young individuals are about 44 micra long by 0.8 micron wide (fig. 35). The average wave length of the spiral is 8 micra with the extremes 6 to 8 micra. The average width of the spiral is 2 micra, with extremes 1.8 to 2.4 micra. These dimensions vary slightly, according to the method used in fixing and staining. The average number of complete turns is 9, with extremes of 5 to 11.

The contours of the cell body are parallel except that the ends taper to sharp points, but no terminal filaments are observed. The chromatin material is arranged in transverse bars equidistant from each other and no specimens were observed in which the chromatin had segregated in wide bars (figs. 34 and 35, plate 4; figs. 38 and 39, plate 5). No polar granules were observed in any of the specimens.

The crista is a narrow, ribbon-like band winding obliquely on the outside of the spirochaete. The margin attached to the cell body is thinner, while the free margin is thicker, as is indicated by the shaded edges in figure 34, plate 4. The crista possesses fine fibrils, which are seen only at the widest portion. In structure the crista resembles the dorsal fin of an eel. The crista in *Cristispira anodontae* has almost as many turns as the cell body, as is shown by the number of visible portions (fig. 34, plate 4). This feature aids in differentiating *Cristispira anodontae* from *Cristispira Balbianii* whose crista has fewer spirals. The crista appears to arise from the cell membrane. It does not reach to the end of the spirochaete, but merges with the cell membrane some distance from the poles.

Cristispira anodontae moves with greater speed than does *Cristispira Balbianii*, exhibiting typical cork screw movements. During retarded motility the spirochaete can be seen rotating on its long axis, although no serpentine contractions and stretchings were observed. Specimens having one end anchored show various twisting and bending movements. *Cristispira anodontae* has no antero-posterior polarity, but move in either direction with equal rapidity.

The method of multiplication was never observed, either in the living or in the stained specimens. On the basis of measurements of the width and length of the cell body, multiplication must take place by transverse division. No specimens were found that were twice as wide as the narrowest spirochaete, while spirochaetes twice as long as the shortest specimens were found. The widest specimen found was one micron wide, while the narrowest was 0.8 micron wide. The longest specimen observed was 88 micra long while the shortest specimen was 44 micra long.

The descriptions of *Cristispira anodontae* vary considerably. Keysselitz described it as having a chambered cell body and pointed ends, but measurements were not given. Fantham described it as 40 micra long by 0.7 micron wide, with extremes of 30 to 60 micra by 0.7 to 1 micron wide, and pointed ends. Schellack described "anodontae" as having rounded ends, the cell body averaging 46 micra by 1 micron with extremes of 39 to 50 micra and 0.9 to 1.2 micron. Schellack has also described another species, *Spirochaeta ostrae*, as averaging 41.5 by 1.1 micra, with extremes 38 to 42 micra long by 1 to 1.3 micron wide. The differences between *Cristispira anodontae* and *Cristispira ostrae* are not sufficient to create separate species. The observations were made by different investigators under different conditions, which may account for the minor variations that are recorded. From these observations and from comparison with the descriptions of similar species, the spirochaete described in this paper resembles Keysselitz's *Spirochaeta anodontae* in the shape of the terminal portions, although differing in the width of the cell body. This spirochaete also resembles Schellack's *Spirochaeta ostrae* in being 0.8 micron wide, but differs in the length of the cell body. Both specimens have a chambered structure and both are found in the intestine of the oyster. It seems clear that this spirochaete belongs to the cristispiras having pointed ends. It is identified as *Cristispira anodontae.*

7. *Cristispira spiculifera* Schellack. This organism was the smallest spirochaete observed during this investigation, and was found only once in an air-dried preparation stained with Giemsa's solution (figs. 8 and 9, plate 1). The contours of the cell body taper down to fine terminal filaments on both ends. Its chromatin material (dark stained portions) is distributed in wide transverse bands. This spirochaete measures 28 to 35 micra long by 0.7 micron wide. The spiral amplitude is 6.4 micra and the width of the spiral is 2 micra.

Since no living specimens were observed, its motility and mode of multiplication were not determined.

While no crista was observed this spirochaete corresponds to what Schellack has named *Spirochaeta spiculifera,* which he found in the crystalline style of *Anodonta mutabilis.* The crista may have been present and failed to take the stain, since no crista was observed on any of the specimens on the same slide.

8. *Cristispira modiolae* Schellack. A spirochaete was found in large numbers in two stained preparations taken from two different oysters. Its outstanding characteristics are: a long spiral amplitude, a wide spiral (figs. 47 to 50); chromatin material shows long dark stained blocks, but generally it is in regular transverse bars: the crista is very narrow and stains light pink, showing no

fibrillar structure. Figures 49 and 50 show normal spirals while figures 47 and 48 show distorted spirals.

The average individual is 58 micra long with extremes of 36 to 78 micra. The width of the cell body is 0.8 micron and occasionally individuals show a width of one micron. The spiral amplitude is 20 micra with the extreme of 16 to 21 micra. The width of the spiral is 5 micra with extremes of 4 to 6.8 micra. The average number of turns is 3 with extremes of 2 to 4 turns. The ends are rounded or bluntly pointed.

This spirochaete corresponds to Schellack's *Spirochaeta modiolae* in having the same width of cell body, rounded ends, no terminal appendages, and narrow crista, but differs in the length of the cell bodies. This spirochaete also resembles, in shape, Schellack's *Spirochaeta tapetos*, but differs in all other points. Had Schellack given measurements of the spiral amplitude, spiral depth, and the number of turns for each organism the comparison would be facilitated. The final classification of this spirochaete may be altered with the accumulation of more data.

9. *Cristispira modiolae* Schellack var. A. nov. var. This spirochaete is similar to *Cristispira modiolae* Schellack, yet the two organisms differ sufficiently to be considered as two varieties. Its outstanding characteristics are a long spiral amplitude, deep spirals, pointed ends, and wide prominent crista (figs. 45 and 46, plate 5). The chromatin is regular, in transverse bars, and polar caps are present.

The average individual is 63 micra long with extremes of 48 to 80 micra for the length. The width of the cell body is 0.8 micron and is constant. The spiral amplitude is 20 micra with extremes of 19 to 24 micra. The width of the spiral is 6 micra with extremes of 4 to 8 micra. The average number of turns is 3 with extremes of 2 to 4 turns.

This sprochaete is similar to *Cristispira modiolae* except that its ends are pointed and its crista is wide and prominent. It further resembles Schellack's *Spirochaeta chamae* but differs from it in length and width of the cell body. There is no spirochaete described that corresponds to this form. It is possible that this spirochaete is the same species as *Cristispira modiolae* Schellack and that the differences may have been due to the long time that the oysters had been out of water when the microscopic observation of these organisms was made.

10. *Cristispira mina* nov. spec. This spirochaete is similar to *Cristispira modiolae* in the form of its cell body, yet its morphological characteristics are half way between those of the cristispiras having narrow spirals and those having wide spirals. *Cristispira mina* (fig. 56, plate 6) was found on two occasions in stained preparations. It has a regular deep spiral with amplitude of medium length which is its chief characteristic. The chromatin material is arranged in regular transverse bars, but no polar caps were observed. The ends taper down to blunt points without terminal appendages. The crista is of medium width, visible at two or three places, showing a fibrillar structure and staining pink with Giemsa's solution.

The average individual measures 54 micra in length with extremes of 38 to 76 micra. The width of the cell body is 0.6 micron with extremes of 0.6 to 0.8 micron. The spiral amplitude is 12 micra with extremes of 9.6 to 12.8 micra. The width of the spiral is 4 micra with extremes of 3.2 to 5.6 micra. The average number of turns is 4 with extremes of 3 to 7 turns.

The mode of motility was not determined because no living specimens were observed. It is probable that *Cristispira mina* moves by rotating on its long axis in a similar manner to that described for *Cristispira Balbianii*.

Multiplication takes place by transverse division as is indicated by the extremes in length of 76 micra for the longest individual and 38 micra for the shortest. No specimens in the process of cell division were observed either living or in stained preparations.

Cristispira mina does not correspond to any other spirochaete that has been described. Its cell body is narrower than that of the other cristispiras, while its spiral amplitude is midway between that of the cristispiras having a long spiral amplitude and that of those possessing a short spiral amplitude. The width of its spiral is half way between the wide and narrow spirals. On the basis of these characteristics the name *Cristispira mina* (a cristispira having a medium spiral amplitude, a medium spiral width, and a narrow cell body) is suggested as descriptive of this spirochaete.

11. *Cristispira tenua* nov. spec. *Cristispira tenua* (figs. 57 and 58, plate 6) is unique in its morphology, resembling *Saprospira lepta* in its terminal portions, but differing from it in possessing a crista. It was found only in one stained preparation. Its cell body is narrow (thin) possessing a short, spiral amplitude, and shallow but regular spirals although often the spirals are distorted as is shown by the middle portion in figure 58. The ends taper down to sharp points, although frequently specimens with blunt points are observed without terminal filaments. The chromatin material shows a tendency to segregate in wide bands, although narrow transverse bars are common. The crista appears to follow the spirals of the cell body, but does not extend to the ends, and the fibrillar structure is not clearly seen.

The average individual is 54 micra long and 0.7 micron wide. The spiral amplitude is 8 micra while the spiral width is 2.4 micra. Since only two organisms were observed the extremes of length and width of the cell body, the spiral amplitude and spiral width could not be determined.

No spirochaete similar to this has been described. On the basis of its narrow cell body, regular spiral, prominent crista, and pointed ends the name *Cristispira tenua* (a thin, narrow cristispira) is suggested as descriptive of this species.

12. *Spirillum ostrae* Noguchi. A slender spiral organism was found in a film from the gut contents of the oyster, in several preparation on several different occasions. Because of its size one is quite apt to overlook this spirillum among the debris generally present in these preparations. This spiral organism possesses 3 to 8 shallow spirals, with the ends tapering to fine filaments (figs. 1 to 6, plate 1). The average spirillum is about 10 micra long with extremes of 7 to 21 micra. The average width of the spiral is about 1 micron. It corresponds to Noguchi's *Spirillum ostrae* which he found in the crystalline style of the oyster.

TABLE 3

Morphological characteristics of spirochaetes and Spirillum ostrae

NAME	SOURCE	LENGTH OF CELL BODY		WIDTH OF CELL BODY		SPIRAL AMPLITUDE		WIDTH OF THE SPIRALS		NUMBER OF TURNS		ENDS	CRISTA
		Average	Extremes	Average	Extremes	Average	Extremes	Average	Extremes	Average	Extremes		
Saprospira lepta	*Ostra virginiana*	70μ	54–92μ	0.5μ	Constant	8μ	5–13μ	1.6μ	1.6–4.8μ	5	3½–10	Pointed	Not present
Saprospira lepta var. A.	*Ostra virginiana*	60μ	45–72μ	0.7μ	Constant	20μ	20–24μ	4μ	3.2–5.2μ	3	2–4	Sharp	Not present
Saprospira puncta	*Ostra virginiana*	80μ	60–100μ	1.0μ	0.9–1.2μ	20μ	16–24μ	6μ	4–8μ	3	2½–6	Pointed	Not present
Saprospira grandis	*Ostra virginiana*	80μ	52–90μ	1.2μ	1.2–1.4μ	24μ	20–28μ	7μ	5–9μ	3½	3–4½	Rounded	Not present
Cristispira spiculifera	*Ostra virginiana*	30μ	28–35μ	0.7μ	0.7–1.0μ	6.4μ	6–7μ	2μ	1.6–3μ	Not determined	Not determined	Pointed filament	Present
Cristispira anodontae	*Ostra virgiana*	70μ	44–88μ	0.8μ	0.8–1.2μ	8μ	6–8μ	2μ	1.8–2.4μ	8	5–11	Pointed	Present
Cristispira Balbianii	*Ostra virginiana*	60μ	39–78μ	0.8μ	0.8–1.0μ	8μ	6–8μ	1.6μ	1.2–2.4μ	7	4½–11	Rounded	Present
Cristispira modiolae	*Ostra virginiana*	58μ	36–78μ	0.8μ	Constant	20μ	16–21μ	5μ	4–6.8μ	3	2–4	Rounded	Present
Cristispira modiolae var. A.	*Ostra virginiana*	63μ	48–80μ	0.8μ	Constant	20μ	19–24μ	6μ	4–8μ	3	2–4	Pointed	Present
Cristispira mina	*Ostra virginiana*	54μ	38–76μ	0.6μ	0.6–0.8μ	12μ	9.6–12.8μ	4μ	3.2–5.6μ	4	3–7	Bluntly pointed	Present
Cristispira tenua	*Ostra virginiana*	54μ	Not determined	0.7μ	Not determined	8μ	Not determined	2.4μ	Not determined	5	4–9	Pointed	Present
Spirillum ostrae	*Ostra virginiana*	10μ	7–21μ	0.4μ	Constant	1.5μ	Not determined	6μ	Not determined	4	3–8	Pointed filament	Not present

DISCUSSION OF SPECIAL POINTS

It has been shown that the apparent morphology of the oyster spirochaetes is influenced by the method of fixation and staining. The best material for the study of morphology was obtained with the wet film preparations.

1. *Meaning of the chambered structure.* The appearance of the chambered structure in *Saprospira Gross* was constant in all the specimens that were examined regardless of the method of preparation. On the other hand the chambered structure in the *Cristispiras* varied with the method of fixation and staining. This variation suggested that the so-called chambered structure was due to coagulation of the cytoplasm into various designs. Hogue (1922) holds a similar opinion in regard to the chambered structure in *Spirochaeta eurygyrata.* Bosanquet (1911) has reached a similar conclusion in his observations on *Spirochaeta anodontae.* Noguchi (1921) is inclined to regard the chambered structure of these spirochaetes as due to the coagulation of the cytoplasm. Gross (1910) interprets the chambered structure of *Cristispira pectinis* as an artefact, resulting from fixation, and he further adds that the cytoplasm of this species is structureless. This view is also supported by the observations in hanging-drop preparations, and dark field illumination.

On the other hand Schellack believes that the chromatin granules are scattered along the inner side of the cell membrane. Dobell is of the opinion that the chambered structure in *Cristispira veneris* is due to transverse chromatin disks, about which the nuclear chromatin is clustered. He compares *Cristispira veneris* to a bamboo pole having the septa at each node.

The correct interpretation of the chambered structure in the oyster spirochaetes probably can not be obtained from stained preparations alone. Experiments with *intra vitam* staining leave no room to doubt the existence of delicate transverse septa in these spirochaetes. It is also evident that the transverse septa, as observed in *intra vitam* preparations, are very thin (narrow), while the transverse bars found in permanent preparations are several times wider than the transverse septa as observed in *intra vitam* staining. The distance between the transverse septa and between the transverse bars is the same as is demonstrated by measurements. It is probable that the transverse septa are the basis (nuclei) about which the transverse bars are built during the staining process. It is not clear whether the transverse bars are made up of the coagulated cytoplasm, whether they consist of chromatin material; or whether they are simply the fixed and stained transverse septa. Dobell's interpretation of the chambered structure, i.e., chromatin granules embedded in the transverse septa, seems most plausible, but he accounts neither for the appearance of the cytoplasm nor for the wide transverse bars in the fixed preparation. The difference in the width between the transverse septa and the transverse bars appears to be due to the cytoplasm adhering to and coagulating about the transverse septa. In imperfectly fixed specimens the chambered structure is irregular as is shown by long blocks of heavily stained areas and wide unstained areas (fig. 7 to 11, plate 1; figs. 13, 15 and 18, plate 2; figs. 28, 32, 36 and 37, plate 4).

2. The crista

The most unique feature of the genus *Cristispira* is the ridge which extends spirally along the cell body but does not reach the poles. This ridge (often called undulating membrane) lead Certes and Perrin to consider these spirochaetes as trypanosomes. Fantham succeeded in demonstrating the fibrillar structure of the crista, while Schellack advanced evidence that the crista is not an undulating membrane, but that it is a true periplast traversed by numerous fibrils. Gross has interpreted the crista not as an undulating membrane but as a ridge winding obliquely to the spirals on the outside of the spirochaete. This interpretation is accepted by the majority of investigators.

The crista is plainly seen in the living cristispira, and its relation to the cell body can be observed. It appears rigid, like a keel, winding spirally about the spirochaete. It serves as a rudder to impart the screw-like movements to the spirochaete. It is very difficult to stain, especially in species other than *Cristispira anodontae* and *Cristispira Balbianii*. The spirochaetes that at present are called saprospira may prove to have cristae with the development of more affective methods of staining.

SUMMARY AND CONCLUSIONS

1. Spirochaetes were found in the digestive tract of 91 per cent of the oysters sold in the markets of Baltimore.

2. These spirochaetes are parasites in the gut of the oyster, and they survive as long as any liquid remains in the digestive tract of the oyster.

3. It is suggested that the classification of the spirochaetes found in the alimentary tract of shell fish be determined according to the presence or absence of crista, the shape of the ends, the width and length of the cell body, the spiral amplitude and the width of the spiral.

4. A spirochaete corresponding to *Saprospira grandis* was identified.

5. Two new species, *Saprospira lepta* nov. spec., and *Saprospira puncta* nov. spec., were found and described.

6. Another saprospira was found closely similar to *Saprospira lepta* and was considered a new variety, *Saprospira lepta var. A.* nov. var.

7. One species of cristispira described in this paper was identified as *Cristispira Balbianii.* The question is raised whether the three species, *Cristispira Balbianii, Cristispira pectinis,* and *Cristispira veneris* are not identical. It is suggested that they be considered as one species, *Cristispira Balbianii.*

8. One species of cristispira was identified as *Cristispira anodontae.* The question is raised whether *Cristispira ostrae* and *Cristispira anodontae* are not identical and it is believed that they could be included under the species *Cristispira anodontae.*

9. A small cristispira was identified as *Cristispira spiculifera.*

10. One species of cristispira was identified as *Cristispira modiolae,* and a similar spirochaete was considered as *Cristispira modiolae var A.* nov. var.

11. Two rather thin cristispiras were considered to be new species and were named *Cristispira tenua* nov. spec. and *Cristispira mina* nov. spec.

12. A slender spiral organism was found in the gut of the oyster and was identified as *Spirillum ostrae.*

13. The opinion is expressed that the appearance of the chambered structure in oyster spirochaetes is due to the coagulation

of the protoplasm about the transverse septa during the fixation and the staining process. The exact interpretation of the chambered structure of these spirochaetes is not at all settled.

14. Evidence is presented to show that the motility of the oyster spirochaetes is caused by the motile "posterior" end portion.

15. Certain observations on the crista suggest that it is a fibrillar structure winding spirally on the outside of the cristispira. It is difficult to stain and shows no independent movement.

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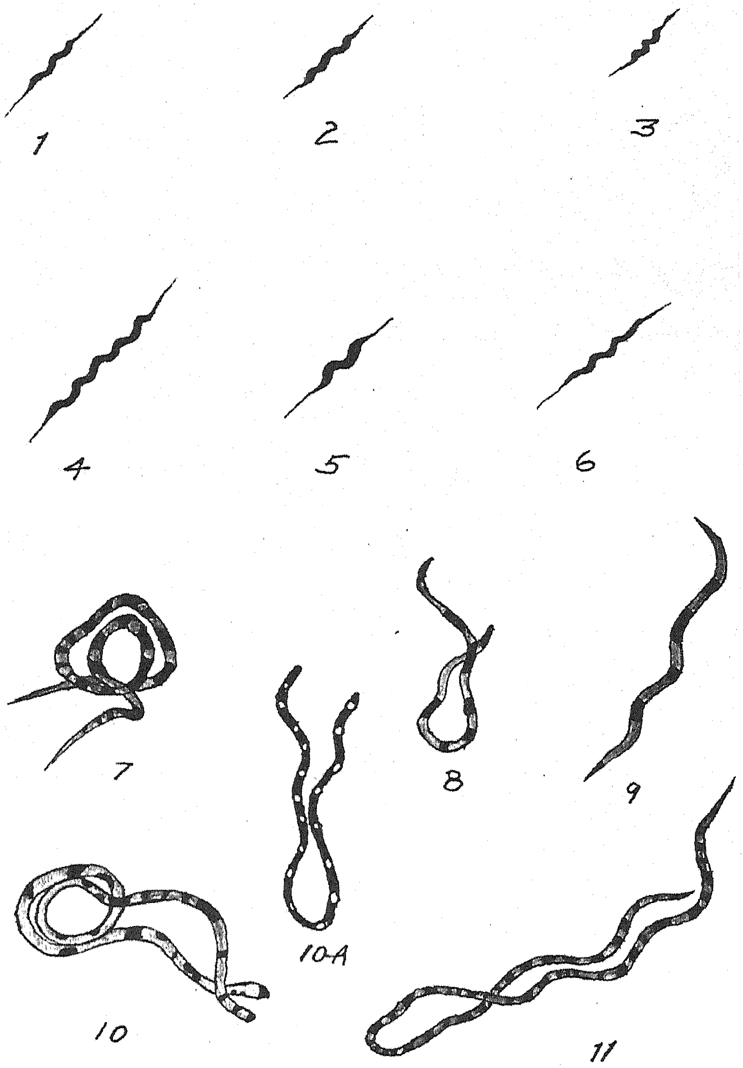
PLATE 1

Camera lucida drawings

FIGS. 1 TO 6. *Spirillum ostrae.* Giemsa's stain.
FIGS. 7 AND 11. *Saprospira lepta.* Giemsa's stain.
FIGS. 10 AND 10a. *Saprospira lepta var. A.* Giemsa's stain.
FIGS. 8 AND 9. *Cristispira spiculifera.* Giemsa's stain.



(Dimitroff: Spirochaetes in Baltimore market oysters)

PLATE 2

Camera lucida drawings

Figs. 12 to 15. *Saprospira puncta*. Giemsa's stain.

Figs. 12 and 14. Taken from moist film preparation.

Figs. 13 and 15. Taken from air-dried preparation.

Figs. 12 to 14. Show constrictions at the wide chromatin bands.

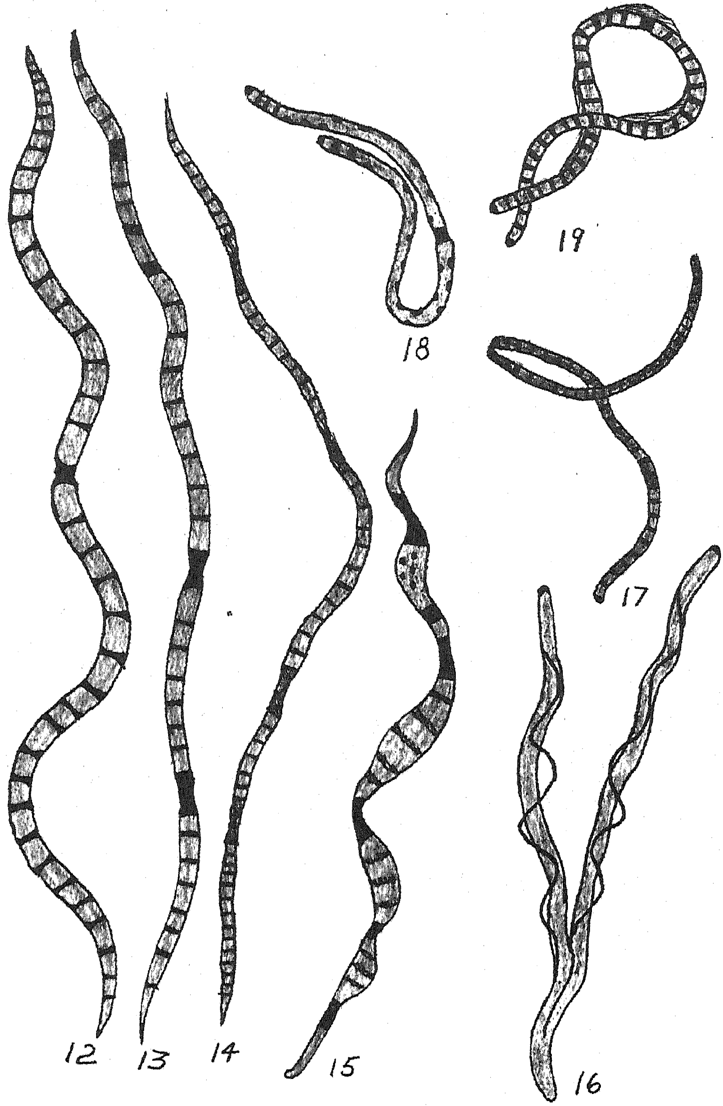
Fig. 15. Shows plasmolysis at the wide portions.

Fig. 16. Dividing *Cristispira Balbianii*. The curved line represents the crista. Carbol fuchsin stain.

Fig. 17. Dividing Saprospira. Giemsa's stain.

Fig. 18. Dividing *Cristispira Balbianii*, but the crista is not shown. Giemsa's stain.

Fig. 19. Dividing *Cristispira Balbianii*, showing the crista. Giemsa's stain.



(Dimitroff: Spirochaetes in Baltimore market oysters)

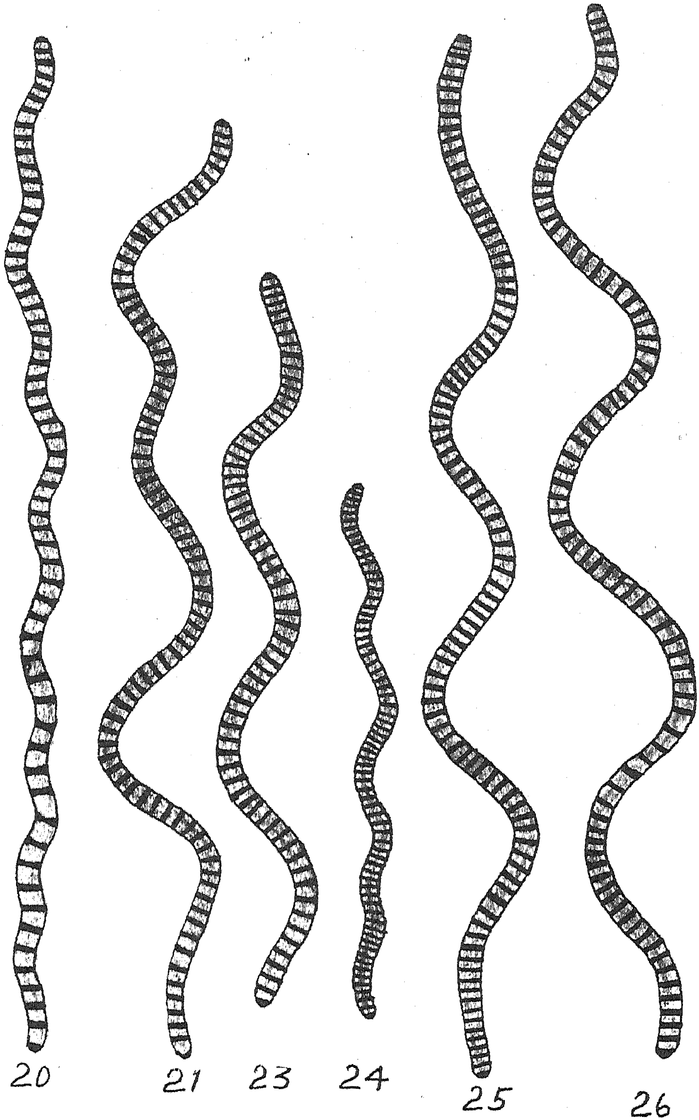
PLATE 3

Camera lucida drawings of *Saprospira grandis*

FIGS. 20 AND 24. *Saprospira grandis* taken from air-dried preparation. Giemsa's stain.

FIG. 21. *Saprospira grandis* taken from moist film preparation. Carbol fuchsin.

FIGS. 23, 25 AND 26. *Saprospira grandis* taken from moist film preparation. Giemsa's stain.



(Dimitroff: Spirochaetes in Baltimore market oysters)

PLATE 4

Camera lucida drawings of Cristispira

FIG. 27. *Cristispira Balbianii.* Moist film. Carbol fuchsin.

FIG. 28. *Cristispira Balbianii* showing the fibrillar structure of the crista and segregation of the cytoplasm into definite areas. Giemsa's stain.

FIG. 29. *Cristispira Balbianii* taken from moist film preparation stained with gentian violet.

FIG. 30. *Cristispira Balbianii* taken from moist film preparation. Giemsa's stain.

FIG. 31. *Cristispira Balbianii* stained solidly by steaming in carbol fuchsin. The curved line represents the crista.

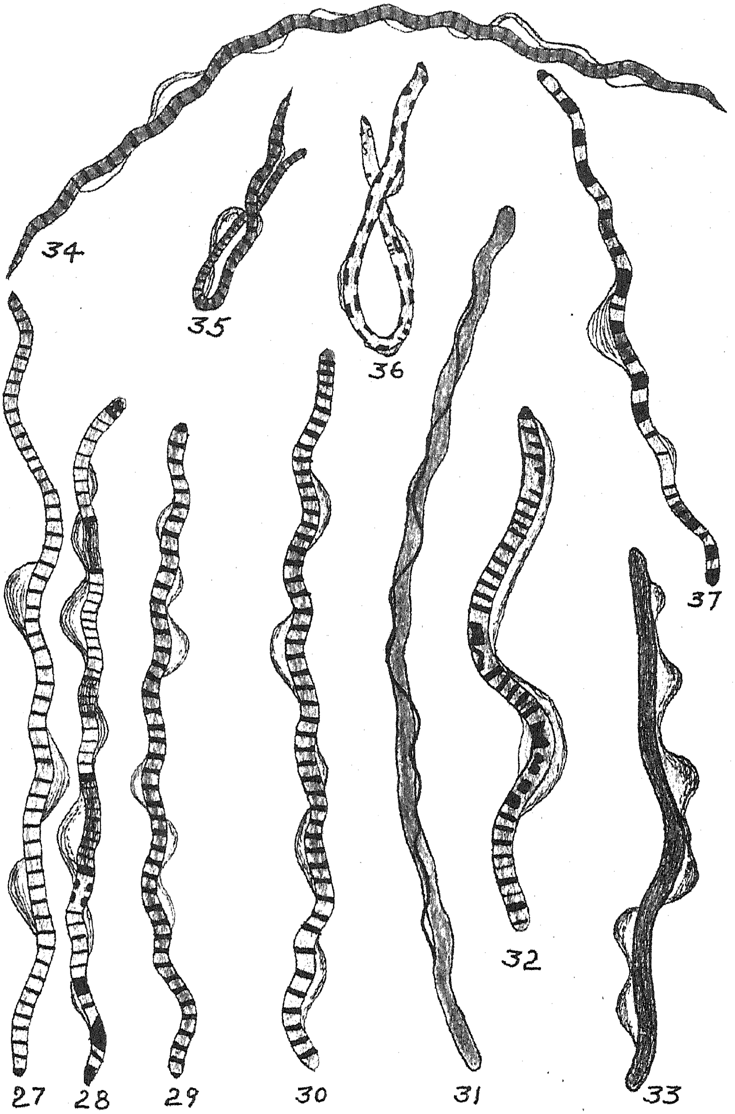
FIG. 32. *Cristispira Balbianii* showing the arrangement of the cytoplasm and the unusual position of the crista. Giemsa's stain.

FIG. 33. *Cristispira Balbianii* representing disintegrating specimen. It is stained solidly and the crista is very wide. Giemsa's stain.

FIGS. 34 AND 35. *Cristispira anodontae* having pointed ends. Giemsa's stain.

FIG. 36. *Cristispira Balbianii* showing the chromatin arranged along the cell membrane. Giemsa's stain.

FIG. 37. *Cristispira Balbianii* showing the chromatin arranged in wide bands, polar caps, and the fibrillar structure of the crista. Giemsa's stain.

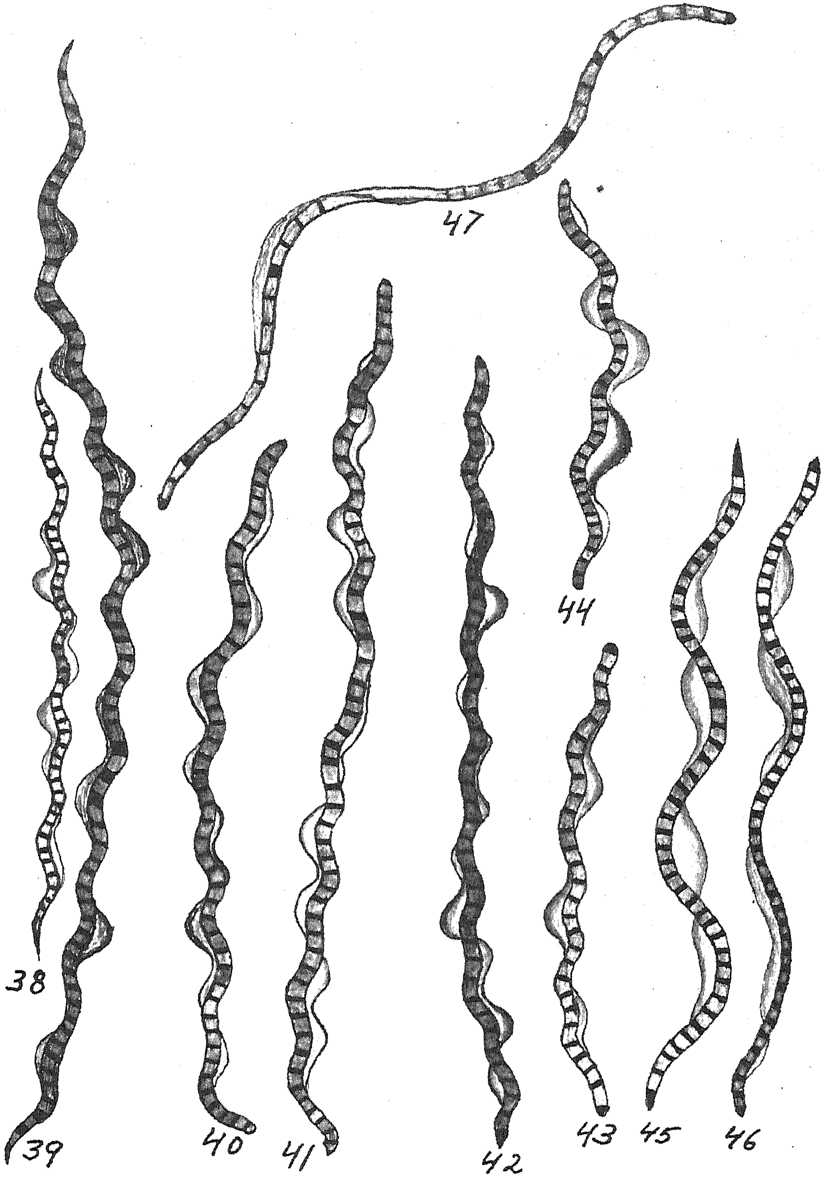


(Dimitroff: Spirochaetes in Baltimore market oysters)

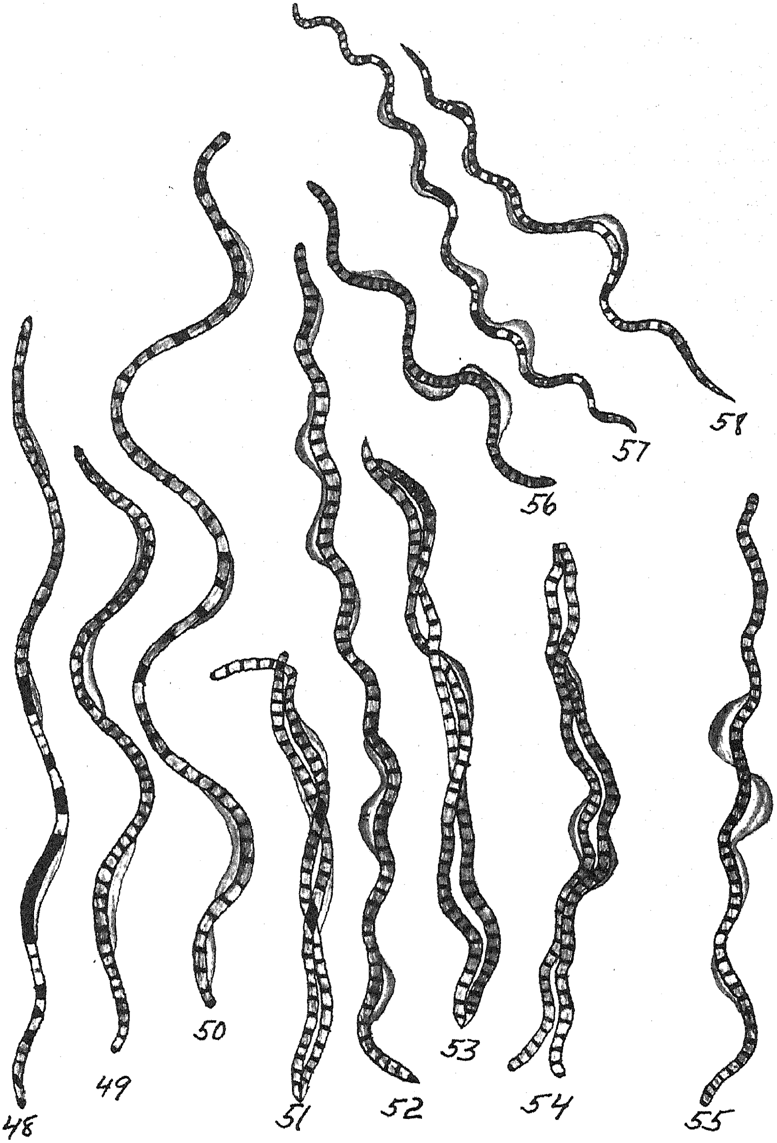
PLATES 5 AND 6

Camera lucida drawings made from moist films stained by Giemsa

FIGS. 38 AND 39. *Cristispira anodontae.*
FIGS. 40 TO 44. *Cristispira Balbianii.*
FIG. 41. Represents an undivided *Cristispira Balbianii.*
FIG. 44. Represents young individual of *Cristispira Balbianii.*
FIGS. 45 AND 46. *Cristispira modiolae.*
FIGS. 47 TO 50. *Cristispira modiolae var. A.*
FIG. 51, 53 AND 54. Dividing *Cristispira Balbianii.*
FIGS. 52 AND 55. *Cristispira Balbianii.*
FIG. 56. *Cristispira mina* nov. spec.
FIGS. 57 AND 58. *Cristispira tenua* nov. spec.



(Dimitroff: Spirochaetes in Baltimore market oysters)



(Dimitroff; Spirochaetes in Baltimore market oysters)

THE EXUDATE FROM NUTRIENT AGAR SLANTS—THE SO-CALLED WATER OF CONDENSATION

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As is well known, a variable quantity of liquid collects in nutrient agar slant tubes,—a condition which does not occur in the case of nutrient gelatin slants. For many years this liquid has been known as the water of condensation, and it is so called in a recent laboratory manual.[1]

As many microbes grow more freely in this so-called water of condensation than they do on the surface of the agar slant or in nutrient broth, it seemed desirable to determine its composition. For this purpose 340 grams of nutrient agar containing 1.5 per cent agar and with a C_H of pH 8.2 were prepared, cut into small pieces, and placed on a large Büchner funnel at 9°C. for four days; during this period, the nutrient agar yielded about 50 cc. of exudate, or water of condensation. This exudate and a sample of nutrient broth were analyzed with the results shown in table 1.

Heat and acetic acid, 1–10, were used to determine the presence of albumen.

Of each sample, 29.5044 grams were evaporated and the residue dried at 105°C. to constant weight for total solids. The residue was burned at a red heat for ash. The ash was dissolved in 1 to 1 HCl and the solution tested for calcium with ammonia and ammonium oxalate; for magnesium with ammonia and sodium phosphate; for phosphorus with ammonia, nitric acid, and ammonium molybdate; for sulfates with barium chloride, and for iron with ferrocyanide solution.

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[1] Laboratory Manual in General and Pathogenic Bacteriology and Immunity. V. A. Moore and W. A. Hagan. 1925, p. 54.

On testing for these minerals the results in table 2 were obtained.

These analyses demonstrate that the liquid which collects in nutrient agar slant tubes is richer in albumen, calcium, magnesium, sulfates, iron, total solids and ash than is nutrient broth, contains phosphates about equal to those of nutrient broth, and is an exudate from the nutrient agar, rather than water of condensation.

TABLE 1

	NUTRIENT AGAR EXUDATE	NUTRIENT BROTH
Color	Light brown	Pale straw
Specific gravity	1.0060	1.0043
Reaction	pH 6.2	pH 7.4
Albumen	Marked, disappears on addition of ammonia	Trace, disappears on addition of ammonia
Total solids	1.61 per cent	1.27 per cent
Ash	0.16 per cent (9.9 per cent of total solids)	0.07 per cent (5.5 per cent total solids)

TABLE 2

MINERAL	NUTRIENT AGAR EXUDATE	NUTRIENT BROTH
Calcium	Marked	Trace
Magnesium	Distinct	Trace
Phosphates	Marked	Marked
Sulfates	Marked	Distinct
Iron	Trace	None

CONCLUSION

The so-called water of condensation which collects in nutrient agar slant tubes is an exudate from the nutrient agar, possessing nutrient substances suitable for sustaining bacterial growth.

A BACTERIOLOGICAL STUDY OF CANNED SALMON

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INTRODUCTION—THE SALMON INDUSTRY

Few consumers of canned salmon are at all familiar with this great North Pacific industry which produces annually in the United States about 7,000,000 cases valued at $55,000,000, and whose product is distributed throughout the world. A very brief summary will be given here in order that the reader may better interpret the experimental data.

There are 5 species of Pacific salmon, the king or chinook, sockeye or red, coho or medium red, pink and chum. All are anadromous, that is, they leave the sea to spawn in fresh water. The parent fish then die, the young usually returning to salt water in their first year. Salmon return after from two to five years, varying with the species, to the very stream as a rule in which they were hatched. At the mouth of the rivers and even far out in the sea, the fish are taken by a variety of gear including fish traps, purse and beach seines, gill nets and troll. The fish are delivered at the canneries by small power boats or in scows and are sluiced or pughed to the cannery floor. The head, entrails, blood and slime are removed, the fish carefully washed, cut crosswise into slices and packed into sanitary cans. These processes are usually carried on either by machines designed for the purpose or by hand. The cans are passed into a steam box for from six to fifteen minutes to remove most of the air, then are tightly closed on the double seamer and finally cooked under steam pressure in huge retorts. For one pound cans the standard cook or process is ninety minutes at 240°F. allowing about five minutes for the retort to reach equilibrium at 10 pounds pressure.

Heat penetration in canned salmon is somewhat slow; for example, this laboratory has proved that a temperature of 212°F. is reached in the center of the can only after thirty minutes exposure at 240°F. In forty-five minutes the center temperature is 220°, after sixty-five minutes it is 230, while after ninety minutes it is only 237°C.

After removing from the retort, the cans are washed in a lye bath to remove oil and particles of fish. At this time leaky cans are recognized by floating or bubbling, and are discarded. After rewashing once or twice in clean water the cans are stacked on the warehouse floor. Lacquer is often applied after an interval followed by labeling. At this time any swells, springers, dented or leaky cans are discarded.

Canned salmon is usually sold by the packer under a guarantee against any and all losses from spoilage. This guarantee is passed along through the broker or jobber to the retailer, who may recover for any spoiled fish or swells which come to his attention.

SAMPLING AND METHODS

Seattle is the headquarters of the salmon industry, fully two-thirds of the salmon produced in the United States and Alaska passing through this city. Samples were usually obtained at the docks or warehouses though many samples were submitted directly to the laboratory by the packers. The product of about 160 canneries, located from Klamath River to the Yukon are represented in this work. In sampling canned salmon, one can is usually taken from every tenth case, though in parcels of over 2000 cases, this proportion is somewhat reduced. The temperature of the salmon ranged from 36° to 65°F. with a mean temperature of about 52°F. The samples were never frozen. When possible they were allowed to stand in the laboratory for several days before examination and if time allowed, a dozen cans were also incubated at 37°F. Probably not over 25 per cent of the cans reported on in this paper were so incubated. Repeated tests upon incubated and non-incubated cans from identical lots always yielded the same results. Because of the low percentage of non-sterile cans found at first, relatively large samples were later examined. The average sample consisted of 18 cans. Extending over a period of five years, 540 separate samples consisting of 7664 cans were examined bacteriologically. These lots consisted of the following species: pinks, 35 per cent; chums, 31 per cent; reds, 15 per cent; kings, 11 per cent and cohoes, 8 per cent. Approximately 80 per cent of the cans were 1-pound talls while the other 20 per cent consisted entirely of ½-pound flats or 1-pound flats.

Two classes or quality grades of canned salmon were artificially made before examination, first, the normal product of unquestioned quality, and second, doubtful quality samples such as lots libeled by the Government or defective in some particular. These classes were made entirely on the basis of the history of the parcel. Swelled or obviously leaky cans were never taken as samples because such cans do not enter the trade. It should be pointed out at this time that salmon libeled as "partly decomposed" by the United States Department of Agriculture has decomposed while in the raw state *previous* to canning. Little active spoilage has been encountered in the canned product.

A detailed organoleptic examination of the salmon, together with observation of the condition of the container, vacuum, and occasional biochemical or microscopic tests, accompanied the bacteriological tests. This was felt to be necessary in order properly to interpret the results. For detailed methods of examining canned salmon see Clark, Clough, Fellers and Shostrom (1923).

MEDIA AND TECHNIC

Before withdrawing samples, the tops of the cans were carefully cleaned with a cloth moistened with 5 per cent phenol, burned for about twenty seconds with the full flame of a Bunsen burner, allowed to cool somewhat and 1 to 2 cc. of 95 per cent ethyl alcohol ignited on the tops. Vacuum tests were made by puncturing the top on the side opposite the vacuum puncture with a sterile awl about the size of a small lead pencil. Large bore pipettes inserted as near as possible to the geometric center if the can were used to withdraw 1-cc. portions of liquor containing small particles of flesh. Such portions were used for duplicate plates, broth and anaerobic plants. Mass cultures of about 10 grams were taken after aseptically opening the can, and transferred to glucose broth. All 7664 cans were tested for aerobes, 1739 of these for anaerobes, 670 for thermophiles, while mass cultures were taken in only 613 of the samples examined.

Standard broth and agar made up according to the American Public Health Association (1920) methods were used throughout the work. Fish, gelatin and sugar agar media were also used for comparison but none gave more uniform results than the standard agar and their use was discontinued in 1921. For anaerobes, lean beef medium with 1 per cent glucose and a small piece of the chopped meat in each tube was used. Media were vaseline stratified and boiled just previous to use. Mass cultures were placed in 250-cc. flasks containing 100 cc. of glucose broth. The usual selective media used for identification were employed in studying the cultures.

TABLE 1

Results of sterility tests on canned salmon

YEAR	NUMBER OF LOTS AND ESTIMATED QUALITY OF SAMPLES EXAMINED BACTERIOLOGICALLY			NUMBER AND STERILITY OF THE SAMPLES EXAMINED BACTERIOLOGICALLY FROM LOTS OF NORMAL QUALITY				NUMBER AND STERILITY OF THE SAMPLES EXAMINED BACTERIOLOGICALLY FROM LOTS OF DOUBTFUL QUALITY				TOTAL NUMBER OF CANS EXAMINED BACTERIOLOGICALLY WITH STERILITY RESULTS (INCLUDES BOTH NORMAL AND DOUBTFUL SAMPLES)			
	Number of lots of normal quality	Number of lots of doubtful quality	Number of lots total	Number of cans examined	Number of cans non-sterile	Per cent non-sterile	Per cent rejected bacteriologically	Number of cans examined	Number of cans non-sterile	Per cent non-sterile	Per cent rejected	Number of cans examined	Number of cans non-sterile	Per cent non-sterile	Per cent rejected
1920	22	54	76	127	22	17.3	8.0	295	42	14.2	3.0	422	64	15.2	4.5
1921	72	42	114	1,008	67	6.7	0.1	546	69	12.6	2.2	1,554	136	8.8	0.13
1922	73	13	86	1,831	31	1.7	0.1	180	15	8.3	0.0	2,001	46	2.3	0.1
1923	98	85	183	1,183	14	1.2	0.2	902	23	2.6	0.4	2,085	37	1.8	0.3
1924	59	22	81	1,127	47	4.2	2.3	475	21	4.4	2.7	1,602	68	4.3	2.4
1920–24	324	216	540	5,276	181	3.4	0.9	2,398	170	7.1	1.6	7,664	351	4.6	1.1

The incubation temperature of 30°C. was arrived at only after several comparative tests using both lower and higher temperatures. Tubes for thermophiles were incubated at 55°C. Usually seventy-two hours was allowed for growth to take place, though anaerobic cultures were not called negative until after seven days. If but one colony was present on a plate or if only one anaerobic tube was positive in three, the results were considered to be negative.

STERILITY OF CANNED SALMON

Table 1 shows the number of lots and separate cans examined by years from 1920 to 1924 inclusive. The sterility of both normal and doubtful quality packs of salmon is tabulated together with the number of rejections on bacteriological grounds.

DISCUSSION

Table 1 shows that of the 540 lots or parcels examined, 324 were normal and 216 of doubtful quality. A total of 7664 cans were examined. Of these 351 or 4.6 per cent were non-sterile and 84 or 1.1 per cent were rejected on bacteriological grounds. The percentage of non-sterility in the canned salmon samples was highest in 1920 and in general decreased to 1924. This is probably accounted for by the excellent educational work carried on by the National Canners Association and Association of Pacific Fisheries as to proper processing methods and by improvement in the sanitary conditions at the canneries. It is seen that the samples of doubtful quality contain a much higher percentage of non-sterile cans than the normal lots. One can only conjecture the reasons for this because both classes received approximately the same process or cook. It may be that the decomposed salmon, containing as it does many more bacteria than the fresh salmon, would leave more survivors in the cans than the latter. We know that initial contamination greatly influences the numbers of surviving organisms and this may be the true explanation.

Approximately 25 per cent of the non-sterile cans were rejected as either undergoing active spoilage or considered unsafe or doubtful as regards future spoilage. It is quite likely that many cans so rejected would have remained perfectly sound. The sterility results in table 1 undoubtedly represent minimum percentages, because in 80 per cent of the cans only 2-gram samples were used for tests and no obligate anaerobic cultures were made on them. On the other hand, in the 1739 cans tested for anaerobes, only 12 (0.6 per cent) were positive where aerobes were not also found. These anaerobes were present as spores which when actually inoculated in sound, well-exhausted cans of salmon would often fail to germinate. For this reason anaerobic tests were employed in a more limited way after 1922. Furthermore true thermophiles were likewise shown to be present in only a negligible percentage (0.9) of the 670 cans examined for them. Mass cultures of 10 grams from 613 cans showed bacteria present in 39 cans or 6.4 per cent non-sterile, a slightly higher figure than the average found for the entire 7664 cans. At any rate, the total percentage non-sterility as reported in table 1 is probably correct to within about 3 per cent.

TABLE 2

Sterility in experimental pack of salmon

SPECIES	NUMBER OF CANS TESTED	NUMBER NON-STERILE	PER CENT NON-STERILE
King	54	4	7.4
Pink	36	5	13.8
Red	48	0	0.0
Coho	48	2	4.1
Chum	60	4	6.6
Total	246	15	6.1

Sterility of experimental packs of salmon

An experimental pack of all 5 species of salmon was made using 246 cans. The fish were taken alive in traps in Puget Sound and some were allowed to stand at a cannery dock for from one to six days before canning. Naturally those canned after the third day were decomposed. The exhaust was twelve minutes at 212°F. while the process or cook was eighty minutes at 240°F. The cans were 1½-pound flats. These canning methods represent average cannery practice. Both aerobes and anaerobes were sought for in these samples. The results may be found in table 2.

The study shows an average of 6.1 per cent non-sterility in the experimental packs canned in the usual commercial manner. This figure checks very well the results in table 1 where 7.1 per cent non-sterility in the doubtful quality salmon was found. It must be borne in mind that nearly one-half of the 246 cans examined in this experiment was more or less decomposed at the time of canning. These data also tend to bear out the point previously considered that such spoiled fish require a longer process to sterilize them. There is no significant variation among the species except in the case of the very fat red salmon where all 48 cans were found to be sterile.

Fiedler (1921) found that 77 per cent of the salmon processed forty-five minutes at 240°F. was non-sterile, 22 per cent after the sixty-minute process, 14 per cent after the seventy-five minute process and 9 per cent after the ninety-minute process. He artificially inoculated fresh salmon of good quality with sporulating bacteria.

Other investigators who have examined canned salmon bacteriologically are the following: Bushnell and Utt (1917) found 52 cans or 100 per cent of various brands to be sterile; Weinzirl (1919) found 2 cans in 17 (11.8 per cent) to be non-sterile, while Savage (1922) obtained the high percentage of 50, though only 16 normal cans were examined. Hunter and Thom (1919) found 44.7 per cent of 530 cans tested to be non-sterile, a total of 94 per cent of these contained a sporing aerobic bacillus. Their samples can scarcely be considered normal quality salmon inasmuch as they were all either actually libeled as decomposed under the Food and Drugs Act or else under suspicion. Similarly, Johnson and Hindman (1913) working with reprocessed goods and "do-overs" found a considerable percentage (not stated) which had large numbers of living bacteria including *B. vulgatus* and *P. vulgaris*. They state such goods are potentially dangerous as food and should not be marketed.

In 34 cans, Cheyney (1919) found 7 (or 20 per cent) contained sporing aerobes only, no other types being isolated.

Houghton and Hunter (1920) examined an unstated number of $\frac{1}{2}$-pound cans of salmon which had been exhausted from seven to fourteen minutes at 210°F. and processed at 242°F. for ninety minutes. All the cans were sterile.

TABLE 3

Group classification of microorganisms in non-sterile canned salmon

ESTIMATED QUALITY OF SALMON	TOTAL NUMBER OF LOTS REPRESENTED	TOTAL NUMBER OF NON-STERILE CANS	NUMBER OF CANS CONTAINING SPORULATING AEROBES		ANAEROBES				COCCI		NON-SPORULATING RODS		TRUE-THERMOPHILES		MOLDS AND YEASTS	
					Facultative		Obligate									
			Number	Per cent	Numbea	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Normal	41	120	97	81	52	43	2	1.6	7	5.8	7	5.8	2	1.6	0	0
Doubtful	36	100	81	81	50	50	3	3.0	9	9.0	9	9.0	4	4.0	1	1.0
Total	77	220	178	81	102	46	5	2.2	16	7.3	16	7.3	6	2.7	1	0.4

TABLE 4

Average numbers of aerobes in non-sterile canned salmon

ESTIMATED QUALITY OF SALMON	NUMBER OF LOTS REPRESENTED	NUMBER OF NON-STERILE CANS EXAMINED	NUMBERS OF AEROBES PER GRAM AGAR 72 HOURS 30°C.							
			Less than 10		10 to 100		100 to 1000		Over 1000	
			Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Normal	50	159	111	70	25	16	16	10	7	4
Doubtful	61	231	143	62	53	23	26	11	9	4
Total	111	390	254	65	78	20	42	10.8	16	4

Numbers and types of bacteria in non-sterile canned salmon

Data were collected to show the relative numbers as well as the general types of microörganisms present in canned salmon. The results are tabulated in tables 3 and 4.

By far the most frequently encountered group was that of the sporulating aerobes which were present in 81 per cent of all the nonsterile cans. Many of these are also classed as facultative anaerobes. The relative numbers of this group per gram of salmon is strikingly shown in table 4. Only small numbers of organisms survive the high processing temperature of canned salmon. For example 65 per cent of the nonsterile cans containing aerobes had fewer than 10 organisms per gram, while only 4 per cent had over 1000 per gram. The sporulating aerobes were numerous in only 2 lots and these were actually undergoing nongaseous spoilage. On the other hand cocci and non-sporulating bacteria were occasionally present in moderate numbers on agar plates. These data indicate that large samples are desirable in testing apparently normal canned foods, otherwise organisms will surely be overlooked. Undoubtedly this very thing occurred in this work where two 1-gram samples were usually taken for aerobes and a similar amount for anaerobes.

Following the sporulating aerobes in numerical importance are the cocci and non-sporulating rods each with 7.3 per cent of the total. We cannot consider the facultative anaerobes as a separate group because they are merely aerobes which are able to develop at very low oxygen tensions. Bushnell (1922) has shown that *Bacillus mesentericus* and other aerobes flourish in the absence of air.

The cocci consisted of 13 micrococci, 4 sarcinae and 1 streptococcus, some occurring in association in the same samples. The non-sporulating rods were a varied lot of organisms ranging from strongly proteolytic types (salmon digestion) to such inert bacilli as *Flavobacterium aurantiacum*. In the light of our present knowledge it is difficult to account for the existence of cocci and non-sporulating rods in canned salmon yet they are found even in cans where the seams are perfectly tight and which have high vacuums. These organisms are all destroyed in from one to five minutes in boiling water, yet through some protective agency in salmon they are able to withstand much higher temperatures. A few undoubtedly gained entrance through leaky seams, though such cans were never used if observed. Initial contamination of the fish, the exhaust, methods of cooking and retort management, cooling and storage may all have a bearing upon the problem.

Obligate thermophiles, as well as obligate anaerobes, are rare in canned salmon, only 2.7 per cent of the former and 2.2 per cent of the latter being found in the samples examined. Many of the common sporulating aerobes grow well at 55°C. but only true thermophiles are meant here. The anaerobes were all clostridia mainly of the welchii-sporogenes type.

Molds were found in only 1 can in 220, but in this particular can spores of *Aspergillus flavus* were abundant. Cocci were also present in the same can which had perfect seams. Neither yeasts nor actinomyces were found.

Species of bacteria in canned salmon

In table 3, a grouping is made of the dominant types of organisms isolated. After placing an organism in a certain group, it was then cultured, purified and kept in stock. After 265 such cultures from 220 non-sterile cans (77 lots), had been collected, an attempt was made to identify as many of them as possible without too much effort. The excellent monographs of Ford (1916) and his associates were used for the sporulating aerobes while Levine (1918) was largely followed for the colon-cloacae group. The works of Conn (1899, 1906), Chester (1900), Bergey (1923), Buchanan (1924), together with many valuable journal articles were also found to be of great service. The excellent Descriptive Charts of the Society of American Bacteriologists proved invaluable in the characterization studies.

The following preliminary classification of the 265 cultures was made:

1. Sporulating aerobes	210
2. Cocci	26
3. Non-sporulating bacilli	16
4. Obligate anaerobes	7
5. Obligate thermophiles	5
6. Molds	1
7. Yeasts and actinomyces	0
Total	265

1. Sporulating aerobes. The technic of Weinzirl (1919) in separating the more important members of this group was followed, the final identification being checked against the descriptions of Ford, Laubach, Lawrence and Rice (1916). Potato and starch agar were found to give excellent satisfaction in separating certain species. Many organisms cannot be definitely assigned to certain species, particularly those of the cereus-vulgatus type. The differentiating characteristics are not well enough marked to make a definite statement as to their identity. For the purposes of this work and to avoid further additions to an already overburdened species terminology, organisms have been placed in the species which they resemble most closely, providing always, of course, that the differences are only slight and that there is fair agreement of important morphological, cultural and biochemical characteristics. Bearing in mind these limitations, the following species were identified.

Bacillus mesentericus Flugge	73
Bacillus vulgatus Flugge	49
Bacillus subtilis (Ehrenberg) Cohn	31
Bacillus cereus Frankland	19
Bacillus circulans Jordan	7
Bacillus cohaerans Gottheil	3
Bacillus megatherium De Bary	2
Bacillus laterosporus Ford	2
Bacillus fusiformis Gottheil	2
Other and unidentified species	22
Total	210

Among the identified species which occur only once are *Bacillus mycoides*, *Bacillus terminalis* and *Bacillus brevis*. The other 19 cultures were unidentified.

2. Cocci. Twenty-six cultures of cocci were studied. These comprised 17 micrococci, 8 sarcinae and 1 streptococcus. *Micrococcus varians* (Dyar) Conn, and *Sarcina lutea* Shröter were each isolated 7 times while a streptococcus morphologically and culturally identical with *Streptococcus pyogenes* Rosenbach was encountered once. When the latter was inoculated subcutaneously in a guinea pig, a large local abscess was formed, from which the organism was re-isolated. This culture is of particular interest because of the prevalence on the Pacific Coast of a streptococcic infection of fishermen caused by handling fresh salmon, halibut, cod or herring. The writer (1921) has often demonstrated the presence of streptococci on raw salmon. Vaughan (1902) has reported the isolation of a toxicogenic micrococcus from canned salmon.

Aside from the specific organisms mentioned above no special effort was made to identify the remaining cultures which were principally chromogens. It is significant that when first isolated most of the cocci showed little or no pigment, whereas after several transplants the pigments became more pronounced.

That certain cocci are very heat resistant is attested by the works of Savage (1922), Weinzirl (1919), and Meyer (1924). All report frequent isolation of cocci from canned foods. These organisms are non-proteolytic and do not form gas. Several cans of sound salmon inoculated with 3 strains for three months showed no visible change in the salmon. Whether their heat resistance is due to the presence of capsular membranes, to the protective colloid action of the salmon, or to the resistance of the vegetative cell itself, has not been definitely settled.

3. Non-sporulating rods. A total of 16 cultures included a variety of these forms. It is difficult to account for their presence in sound canned salmon, particularly in view of the fact that all samples examined were entirely normal in appearance. Some may have gained entrance into the cans through very small openings in the seams, though this does not appear likely in most cans inasmuch as these samples carried an average vacuum of 8.1 inches of mercury. The percentage of cans showing less than 3 inches of vacuum was 9.1 per cent. Manifestly a certain degree of under-processing must have occurred in the case of at least a few of the lots. The following species were identified.

Zopfius zopfii (Kurth) Wenner and Rettger	1
Alcaligines fecalis (Petruschky) Castellani and Chalmers	2
Chromobacterium janthinum Hoff	1
Flavobacterium aurantiacum Frankland	1
Aerobacter cloacae Jordan	1
Achromobacter non-liquefaciens (Frankland)	2

Pseudomonas non-liquefaciens (Eisenberg)	2
Unidentified (non-characteristic colorless rods)	4
Total	16

These organisms are principally water bacteria and their presence indicates little save either serious under-processing or faulty seams in the cans where they were found. Since it is known that no obvious defects were present in the seams, the presence of these non-heat resistant bacteria must be accounted for by under-processing. Only one, *Aerobacter cloacae* is capable of forming gas yet the can from which it was obtained was quite normal. This group of organisms is characterized by general inactivity so far as proteolytic action upon salmon flesh is concerned. It is significant that neither *Escherichia coli* nor *Proteus vulgaris* were isolated from any normal cans, though both were frequently found in swells. This fact probably explains the dearth of gas-forming bacteria identified in this study. In other words swells and leakers caused by such organisms were always discarded at the time of sampling and were not cultured.

4. Obligate anaerobes. Only 7 cultures were obtained, and 2 of these died out before they could be identified. In two cases *Zopfius zopfii* was associated with the anaerobe, in another case *Micrococcus varians,* while in 4 cases the anaerobe was the only organism present. Gaseous decomposition was not evident in any of the above 7 cans. It was not considered possible at first for anaerobes to be present in well exhausted canned salmon without producing gas or putrefaction, but this view was soon found to be untenable. Savage (1922) has likewise pointed out that spores of anaerobes may live in sound canned foods without showing growth. He states:

> While it appears to be established that obligate anaerobes can live in the complete absence of oxygen it would appear that growth is better when a very small amount is available. . . . We would advance the view that anaerobes are probably present in considerable numbers in a considerable proportion of these marine products. When in large numbers they may be able to produce sufficient enzymes to break down enough protein to enable them to grow still more and ruin the food. On

the other hand when present in small numbers, and invariably as spores, the nutrient material round them is not sufficiently broken down to enable them to develop and they remain dormant as spores. Given conditions such as those described above, which supply the simpler products they need, they will multiply and decompose the food.

Whether or not this view is tenable, it readily explains his results as well as those reported in the present paper. The organisms identified were *Cl. butyricum* Prazmowski 2, *Cl. welchii* (Welch and Nuttall) 2, and *Cl. sporogenes* Metchnikoff 1.

From these results it does not appear that the obligate anaerobes are very abundant in sound canned salmon. The contention of Savage that anaerobic spores may be present in sound canned foods without producing decomposition is verified.

5. Obligate thermophiles. Only 5 cultures out of the 265 proved to be true thermophiles, and 2 of these showed scanty growth at 37.5°C. but none at 30°C. after several transfers. One of the cultures was identified as *Bacillus aerothermophilus* Weinzirl, the others were not definitely assigned to any species. All four formed spores of greater diameter than the rods, were motile, non-proteolytic and caused no changes in good canned salmon when inoculated into it. On the other hand *Bacillus aerothermophilus* after three months caused softening of the fish together with a disagreeable odor and sour taste. No gas was formed. On the whole this group appears to be of little significance in canned salmon either from the standpoint of numbers or action in this product.

It should be recalled at this place that many strains of the sporulating aerobes such as *Bacillus mesentericus*, *Bacillus subtilis* and *Bacillus vulgatus*, apparently readily acquire or lose the ability of growth at high temperatures. Many thermophiles isolated by the writer from dust in empty cans were common sporulating aerobes which grew at 55°C. as well as at 30°C. This point has also been considered by Savage (1922) who likewise showed that many common aerobes grow readily at 55°C.

6–7. Molds, yeasts and actinomyces. Only one mold was proved to be present in canned salmon. This was *Aspergillus flavus* and was not growing actively in the can. Several leaky cans of salmon were found with heavy growths of *Penicillium* but these were undoubtedly introduced from the air subsequent to processing. It is well known that smoked or dried salmon molds very readily. No yeasts or actinomyces were found in any of the canned salmon examined. Manifestly molds, yeasts and actinomyces are rarely encountered and of little significance in sound canned salmon.

Inoculation experiments with canned salmon

In order to ascertain the action of some of the bacteria isolated from canned salmon in the sound canned product, a number of experimental inoculations were made. Chum salmon from a lot which showed no insterility was used. Furthermore, at the time of inoculation, bacteriological samples were withdrawn from all cans to make certain that contaminating organisms were absent. Only 7 cans in 97 showed the presence of foreign organisms and these were discarded. The cans were incubated from one-hundred and forty to one-hundred and forty-seven days at summer laboratory temperature (23°C.) The cans were exhausted by heating in a pressure cooker at 100°C. for twelve minutes, inoculated in the center and immediately sealed. The inoculum consisted of 0.2 cc. of a five-day broth culture. The final count of organisms was made on agar after seventy-two hours. Direct microscopic examination was used to observe the presence of active vegetative cells. The odor of the salmon was tested by at least 2 persons in every case.

The data in table 5 show that in general the sporulating aerobes are not able to cause marked spoilage in canned salmon, though most species make active growth. An abnormal odor, not always well marked or definitely disagreeable is the usual result of the growth of these organisms in non-exhausted cans of good salmon. The odors were usually classed as sour, or clammy, or as resembling seaweed or raw fish. In almost every case the odor and soft texture were more marked in the cans which had not been exhausted. There was a minimal growth in cans having high vacuums of from 15 to 20 inches showing that most of these organisms are facultative anaerobes. The relative numbers of

TABLE 5

Inoculation experiments with canned salmon

SPECIES TESTED	NUMBER OF CANS	CANS EXHAUSTED	TIME INCUBATION AT 23°C.	VACUUM WHEN TESTED	FLESH ODOR	FLESH TEXTURE	FLESH REDDENED	TURBID LIQUID	ORGANISM RECOVERED	VEGETATIVE CELLS OBSERVED	AVERAGE BACTERIA PER GRAM
			days	*inches Hg*							
B. aerothermophilus............	3	No	145	0	Slightly stale	Slightly soft	No	Yes	Yes	Few	80,000
	3	Yes	145	7	Normal	Firm	No	No	Yes	None	70
B. albolactis....................	1	No	145	12	Slightly sour	Slightly soft	No	Slightly	Yes	Many	1,000,000
	1	Yes	145	3	Normal	Slightly soft	No	Slightly	Yes	Few	600
B. cereus.......................	5	No	143	1⅓	Slightly abnormal like clams	Soft	Slightly	Yes	Yes	Many	360,000
	3	Yes	143	11½	Slightly abnormal like raw fish	Slightly soft	No	Slightly	Yes	Many	14,000
B. circulans....................	2	No	143	1⅓	Very slightly abnormal, sour	Slightly soft	No	Slightly	Yes	None	3,500
	3	Yes	143	13½	Normal	Firm	No	No	Yes	None	200
B. cohaerans....................	1	No	141	½-P*	Slightly abnormal	Slightly soft	No	Slightly	Yes	Many	5,000,000
	1	Yes	141	3	Very slightly abnormal; lack of odor	Firm	No	Slightly	Yes	Moderate	50,000
B. fusiformis..................	1	No	141	0	Normal	Firm	No	No	Yes	Few	30,000
	1	Yes	141	8	Normal	Firm	No	No	No	None	0
B. mesentericus...............	11	No	140–143	2⅓	3 normal; 8 abnormal—sour	Slightly soft	2 slightly	Slightly	Yes	Many	1,200,000
	8	Yes	140–143	15	4 normal; 4 lack of odor	Slightly soft	No	Slightly	Yes	Very few	7,800

B. subtilis....................	4	No	141–144	2	Abnormal; slightly sour	Slightly soft	2 bleached	Yes	Yes	Many	70,000
	5	Yes	141–145	17	Slightly abnormal; lack of odor	Normal	No	No	Yes	Very few	3,200
B. vulgatus....................	3	No	143	0	2 abnormal; slightly sour	Soft	2 slightly	Slightly	Yes	Few	3,000,000
	3	Yes	142	18	2 slightly abnormal	Slightly soft	No	No	Yes	Few	3,900
S. schotmülleri................	3	No	140	7–P	1 slightly abnormal	Slightly soft	No	Yes	Yes	Many	110,000,000
	3	Yes	140	12–P	2 slightly abnormal	Slightly soft	No	Yes	Yes	Many	13,400,000
Aerobact. cloacae..............	2	No	141	18–P	Tainted	Slightly soft	Yes	Yes	Yes	Many	3,900,000
	2	Yes	141	9–P	Tainted	Slightly soft	Yes	Yes	Yes	Many	6,000,000
Mic. varians..................	2	No	147	2	Normal	Firm	No	No	Yes	Few	2,700
	3	Yes	147	19	Normal	Firm	No	No	Yes	Few	3,600
Cl. botulinum A..............	6	No	7	18–P	Putrid	Liquid	No	Yes	Yes	Many	About 70,000,000
	4	Yes	7	12–P	Putrid	Liquid	No	Yes	Yes	Many	About 50,000,000
Thermophiles (10 strains).....	10	Yes	92	10	Normal	Firm	No	No	Yes	No	No test made

* P = lbs. pressure per sq. in.

bacteria in the exhausted and non-exhausted cans show striking differences. The numbers in the former are often from 100 to 1000 times greater than in the latter. No gas was formed in any case, the average composition of the headspace gases of 13 cans being: CO_2, 16.7 per cent; nitrogen, 83.3 per cent. No oxygen was found in any case. It is of interest to note that *Cl. botulinum* A causes hard swells, liquefaction and an intensely putrid odor in either exhausted or non-exhausted canned salmon. Little visible action was caused by *Micrococcus varians* or any one of a series of 10 thermophiles. These organisms were originally isolated from water by Professor Tanner of the University of Illinois, who kindly furnished the cultures. Another thermophile, *B. aerothermophilus* caused some softening of the texture and an abnormal somewhat sour odor. *Aerobacter cloacae* produced gas and a disagreeable odor but otherwise had little effect opon the salmon.

Canned salmon and food poisoning

Geiger, Davis and Benson (1924) inoculated several members of the Salmonella group into canned salmon and found that all made a vigorous growth while all except *Salmonella enteritidis* formed gas. In view of these findings and because of the low heat resistance of these organisms there is perhaps only a slight danger of poisoning from this group of bacteria. Savage (1923) on the other hand cites 14 cases of food poisoning from canned salmon, but in only one case were the causative organisms or their toxins actually demonstrated. He believes that salmon may become infected with *Salmonella aertrycke* or similar organisms before canning, the toxins being able occasionally to withstand the processing temperature.

Geiger (1923) investigated epidemiologically 149 cases of alleged food poisoning from canned salmon, but in no case was the causative organism or toxin experimentally proved. He makes the point that if all these reported outbreaks were actually caused by canned salmon the poisoning incidence in this product from 1910 to 1922 would be one case in 2,500,000 cans. He states:

> There is a tendency to incriminate commercially canned foods. Such foods have the advantage over other types of food by being relatively free from human contact, usually prepared in sanitary plants and from fresh material. But most important there is properly controlled sterilization based on scientifically acquired data. Therefore contamination by the paratyphoid group is unlikely in such foods except in leaky cans and after opening.

Jordan (1917) likewise states:

> Proper investigation of an outbreak (food poisoning) is so rarely carried out that the articles of food ordinarily accused are selected rather as the result of popular prejudice and tradition than of careful inquiry.

So far as the writer is aware *Clostridium botulinum* has never been isolated from commercially canned salmon, nor has botulism been attributed to this product. An outbreak of fowl botulism caused by infected and spoiled home canned salmon has been reported by Geiger, Dickson and Meyer (1922).

From the evidence of others as well as from the data presented in this paper, it appears that ordinary sound canned salmon is likely to be only rarely a carrier of food infections.

SUMMARY

In a five-year period 7664 cans of salmon comprising 540 separate lots were bacteriologically examined. The technic and media employed are described. Of the total cans, 4.6 per cent were found non-sterile and 1.1 per cent were rejected for bacteriological reasons.

The samples were classified into two grades, first sound normal canned salmon and second, doubtful lots such as libeled, reprocessed, reconditioned or questionable fish. No swells or leaky cans were considered in either class. In 5276 cans of sound, normal salmon 3.4 per cent were non-sterile with 0.9 per cent rejected while in 2398 cans of doubtful quality salmon 7.1 per cent were non-sterile with 1.6 per cent rejected on bacteriological grounds.

A considerable improvement is noted in the percentage insterility from 1920 with 15.2 per cent to 1924 with 4.3 per cent. This is probably due to improved and standardized methods of processing and advancements in cannery sanitation.

Insterility in itself is not a sufficient basis for rejection of canned salmon. The vacuum, seams, character of the organisms and probably storage conditions must be considered as well.

The per cent insterility of 246 $\frac{1}{2}$-pound flat cans including all five species of salmon and processed uniformly at 240°F. for eighty minutes, was 6.1.

Out of 220 non-sterile cans of salmon from 77 lots the following number of cans were found to contain certain bacterial groups, i.e., sporulating aerobes 178 (81 per cent); cocci 16 (7.3 per cent); non-sporulating rods 16 (7.3 per cent); obligate anaerobes 5 (2.2); true thermophiles 6 (2.7 per cent); molds 1 (0.4 per cent).

Seventy per cent of the cans containing aerobes showed less than 10 bacteria per gram.

Over 50 per cent of the sporulating aerobes grew in canned salmon and on laboratory media under nearly perfect anaerobic conditions. From 265 consecutive cultures obtained from 220 non-sterile cans, 210 were sporulating aerobes, 26 cocci, 16 non-sporulating bacilli, 7 obligate anaerobes, 5 true thermophiles, 1 mold and no yeasts or actinomyces.

Of the sporulating aerobes *Bacillus mesentericus* Flugge was most frequently encountered with 73 isolations while *Bacillus vulgatus* Flugge, *Bacillus subtilis* Cohn and *Bacillus cereus* Frankland followed with 49, 31, and 19, respectively. Eight other species occurring in lesser numbers were also identified.

Seventeen micrococci, 8 sarcinae and 1 streptococcus were isolated, the most common cocci being *Micrococcus varians* (Dyar) Conn and *Sarcina lutea* Schröter. *Streptococcus pyogenes* Rosenbach was isolated once.

The 16 non-sporulating bacilli were a varied lot with few characteristics in common. *Zopfius zopfi* (Kurth) Wenner and Rettger, *Aerobacter cloacae* Jordan and a number of common water bacteria were found. Neither *Escherichia coli*, *Proteus vulgaris* nor any member of the Salmonella group was found in either sound or doubtful quality canned salmon.

Only 7 obligate anaerobes were found. *Clostridium butyricum* Prazmowski, 2; *Clostridium welchii* Welch and Nuttall, 2; and *Clostridium sporogenes* Metchnikoff, 1, were identified. They were present in canned salmon as spores only.

Obligate thermophiles were encountered only five times. They are of little significance in canned salmon. *Bacillus aerothermophilus* was the only species definitely identified. Many of the common sporulating aerobes are facultative thermophiles.

Yeasts, molds and actinomyces are very rare in sound canned salmon.

Inoculation experiments show that many sporulating aerobes grow well in canned salmon but are not able to cause gas production or marked spoilage. A softening of the texture of the flesh, an abnormal odor and sometimes discoloration, results. A minimal growth of many sporulating aerobes occurs in even well exhausted cans of salmon. A vigorous growth often takes place in non-exhausted cans, in spite of the presence of only traces of oxygen.

Clostridium botulinum grew vigorously in canned salmon even at low temperatures with profuse gas formation, putrefaction and liquefaction of the salmon. It has never been found in commercially canned salmon and no outbreaks of botulism have been attributed to this food. Thermophiles and cocci showed little action when inoculated into sound canned salmon.

The Salmonella group was absent and judging from the characteristics of this group and the lack of evidence implicating canned salmon it is believed that food poisoning from this source is very rare.

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BACTERIOPHAGY IN URINARY INFECTION[1]

PART I. THE INCIDENCE OF BACTERIOPHAGE AND OF BACILLUS COLI SUSCEPTIBLE TO DISSOLUTION BY THE BACTERIOPHAGE IN URINES. PRESENTATION OF CASES OF RENAL INFECTION IN WHICH BACTERIOPHAGE WAS USED THERAPEUTICALLY

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INTRODUCTION

The extensive and rapidly increasing literature on the subject of bacteriophagy contains but few instances of a complete and thorough investigation of the rôle played by the bacteriophage in a given disease. No investigator, with the exception of the discoverer of the phenomenon of bacteriophagy, d'Herelle, has attempted to ascertain the exact conditions obtaining in a localized infection with respect to the bacteriophage or to the type of bacterium encountered, although in his studies of typhoid fever, barbone and fowl typhoid he has indicated in a very positive manner the value, as measured by results, of an extensive inquiry into the conditions prevailing in a particular infection. That no one has elected to follow d'Herelle's lead is perhaps not surprising in view of the enormous number of attractive problems resulting from the discovery of the phenomenon of bacteriophagy. Therapeutic work with the bacteriophage undertaken without the knowledge gained by studies such as have been made on typhoid fever, barbone and fowl typhoid must, however, be at best empirical. Realizing the importance of a knowledge of the conditions prevailing in a given disease as a basis for therapeutic experiments with bacteriophage, an investigation of infection of the urinary tract has been undertaken; the results of which will be presented in a series of papers.

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[1] This paper forms part of a thesis presented for the Degree of Doctor of Philosophy in Yale University.

The present contribution is concerned with a statement and consideration of the results obtained from a systematic search for races of bacteriophage in urines of infected and non-infected individuals, and from a study of the state of the colon bacilli present with respect to their degree of susceptibility to dissolution by the bacteriophage. Subsequent papers dealing with the action of the bacteriophage in the body, and the effects of tissues and excretions, especially the urine, upon the bacteriophage, will be presented. The results provide some information as to the occurrence, permanence, source and fate of the bacteriophage in the urine, and the character and behavior of the *Bacillus coli* concerned in urinary infections. Such a study should contribute materially to a better conception of the rationale of bacteriophage therapy in pyelitis, cystitis, and associated infections.

PROCEDURE

In order to detect the presence of bacteriophage in a given medium, it is necessary that there should be provided a bacterium capable of undergoing dissolution as a result of the activity of the bacteriophage. Lysis, as it has been termed, whether evidenced by inhibition of bacterial growth, clearing of a turbid culture, or the formation of plaques on solid media, is the only criterion at present available for the detection of bacteriophage. When, after the contact of material in which bacteriophage is sought with a bacterial strain, lysis is found to occur, and when this lysis is reproducible in series, the conclusion that bacteriophage is present is justified. On the other hand, the failure to obtain such evidence of its activity is never proof that the principle is absent. It can only be said, that bacteriophage active for the bacterial strain provided and of sufficient virulence to display its activity, is not present. In searching for bacteriophage in a medium in which its presence is suspected, however, the greater the number of bacterial strains provided, and the more numerous the contacts, after successive filtrations, the more likely becomes the conclusion, when no activity is apparent that no bacteriophage is present. At best, however, negative results can ill support interpretation, although positive findings provide a safe basis on which to build.

In searching for races of bacteriophage in urines, a technique was devised which provided, insofar as was possible in a routine study, the conditions most favorable for the detection of bacteriophage. Urines from patients with infections of the urinary tract were incubated for 18 hours at 37°C. after the addition of alkaline extract broth[2] the proportion of broth to urine being about 1:1. When, after eighteen hours of incubation, growth was apparent, and when smears proved that the organism was a Gram-negative bacillus, subcultures were made by streaking Endo plates, and the original culture subjected to filtration. The filtrate was the medium in which bacteriophage was sought.

To tubes of alkaline extract broth containing about 10 cc. of medium, these filtrates were added in amounts of 1, 0.5, and 0.1 cc. To the fourth tube no filtrate was added. Each of the four tubes was then inoculated with a drop of a suspension of *Bacillus coli*[3] which has been found to be susceptible to dissolution by a number of filtrates containing bacteriophage. This strain of *Bacillus coli* was, then, the indicator. As has been explained, bacteriophage might be present without having an activity for this particular colon bacillus, for d'Herelle (1924) has shown that *Bacillus coli* is an heterogeneous species insofar as susceptibility to the dissolving action of bacteriophage is concerned. Consequently, it is quite apparent that one strain of *Bacillus coli* is not a sufficient indicator. In order to increase the chance of obtaining a positive result, a second series of tubes was prepared, identical with the first except for the bacterium used. In this instance the strain of *Bacillus coli* isolated from the patient's urine, if such a strain was available, was utilized. A third series was arranged in which the bacterium used as indicator was *Bacillus dysenteriae* Flexner which experience had shown to have a wide range of susceptibilities including susceptibility to most races of colon-bacteriophage. Immediately after the inoculation, a loopful of the material from each tube in each series was streaked on agar slants, and both the slants and the broth tubes were incubated at 37°C. for eighteen hours. Readings were made at various intervals from three to eighteen hours.

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[2] Alkaline extract broth was used in all experiments dealing with the bacteriophage. It was made by adjusting the reaction of plain extract broth to a pH of 7.8, which concentration of hydrogen ions has been shown to be favorable to the manifestation of bacteriophage activity.

[3] Throughout these experiments, the suspensions of *Bacillus coli* used to inoculate the tubes was obtained by washing the growth from the surface of an eighteen-hour slant with 10 cc. of alkaline extract broth.

As has been stated the action of bacteriophage on a bacterium may be indicated by inhibition of growth, dissolution of a growth sufficient to cause turbidity in broth, or the formation of plaques. In the event of the dissolution or inhibition, the result is noted by comparison with the control tube in which a normal culture of the bacterium has been inoculated. The degree of turbidity in the broth may vary from a state scarcely to be differentiated from the control to one of complete limpidity. In making readings then, it is sometimes convenient to note the degree of activity of the bacteriophage. Although the analyses to be made in the present instance depend upon the presence or absence of the bacteriophage and not upon the degree of virulence manifested, the readings are presented as they were made. A completely limpid tube indicated the presence of bacteriophage of activity noted as ++++, while slight turbidity, but markedly less than the control was regarded as evidence of +++ activity. The agar slants corresponding to the tubes in which such readings were obtained remained sterile. Bacteriophage of low virulence was recorded as of ++ activity when there was only a very slight difference between the tube containing the filtrate and the control, and as of + when the appearance of plaques on the agar slants was the only evidence of the presence of the lytic substance.

There is no necessity for including in the table of results of the above investigation a statement of the further steps which must be undertaken to verify the existence of a true bacteriophage. It is well known that the phenomena of bacterial dissolution and of plaque formation have been observed as resulting from substances undoubtedly not bacteriophage. As examples Flemming's lysozyme, Twort's bacteriolysin, and the pyocyaneus "bacteriophage" of Hadley might be cited. Serial activity, when demonstrated together with plaque formation and bacterial dissolution in broth are, however, the criteria which serve to identify bacteriophage. Serial activity was demonstrated with a considerable proportion of the active filtrates in this series, the number of passages varying from 2 to 10.

It is further to be noted that repeated contacts of a filtrate in which one is seeking evidence of bacteriophagic activity, with a susceptible bacterium, each contact secured by a separation of bacterium and fluid through filtration and the subsequent use of the subculture of the bacterium which has not been in previous contact with the bacteriophage, may result in enhancing the virulence of any bacteriophage present to such an extent that its activity becomes apparent. If after several contacts there is no evidence of bacteriophage action, it is safe to conclude that the filtrate does not contain the agent in a form in which its activity will ever become apparent. Obviously in routine procedures it is extremely tedius to employ such a method. Nevertheless, when the conclusions are based upon the absence of bacteriophage, no other method is permissible. On the other hand, when the presence of bacteriophage is the basic condition, failure to demonstrate it after one contact does not lead to serious errors in the results. In the present instance, when absence of bacteriophage was the important consideration, successive contacts were employed, but in the opposite condition a single contact only was made.

As a corollary to the search for bacteriophage in urines, a study of the *Bacillus coli* encountered was made to determine whether the bacterium was susceptible to the action of bacteriophage or whether it was resistant. To this end a procedure similar to that already described was followed. The conditions were reversed however. Two filtrates were placed in contact with the organism isolated from the urine. The first filtrate was known to contain a highly virulent bacteriophage for several strains of *Bacilli coli*. The second was the filtrate of the urine from which the colon bacillus was isolated. In obtaining the bacterium from the urine, single colonies were taken from the Endo plates which had been streaked when the urine was first cultured, and alkaline agar slants inoculated. The procedure and methods of reading were identical with those described for the detection of bacteriophage.

Table 1 presents the results of these studies together with some related data, the significance of which will appear later. Although 102 tests are shown in this table, more than 200 urines were studied. The tests not recorded were made on specimens of urine obtained by catheterization of the ureters at the same time that bladder specimens were obtained. In every case the results of the tests of kidney urines were identical with those of the specimens secured from the bladder. Such tests were not without significance, however, for they indicated that the conditions in the kidney pelves were identical with those found to exist in the bladders insofar as the activity of the bacteriophage and the type of the bacterium were concerned.

The diagnoses in every case were made by the attending clinician and were obtained from the case histories. When several tests are described for the same patient these tests were made on specimens obtained at different times and the results are arranged in chronological order.

It is evident from the preceding table that we have six possible conditions with relation to the incidence of bacteriophage and the type of *Bacillus coli* in the urine. One can consider the presence of bacteriophage when susceptible colon bacilli, resistant colon bacilli and no bacteria, respectively, are found, and, on the other hand, the incidence of the two types of *Bacillus coli* in the absence of bacteriophage. The sixth possible condition is the absence of both bacteria and bacteriophage which is the condition in normal urines. It is likewise obvious that two means of interpretation of the results are available since two different units may be considered. Urines, regardless of the individuals from whom they were collected, may be studied, or, one may consider only the individuals, without reference to

TABLE 1

CASE DESIGNATION	SEX		URINE LYTIC FOR			TYPE OF B. COLI PRESENT
			S	F	H*	
BK	F.	Pyelitis-cystitis, acute and chronic	–	–	–	R
			–	–	–	R
			+++	0	++++	++++
			++	–	–	R
			–	+++	–	R
			–	–	–	R
LS	F.	Pyelitis-cystitis, acute—abortion (therapeutic)	++++	++++	++++	++++
LB	F.	Pyelitis-cystitis, acute and chronic (pregnant)	–	–	++++	++++
			++++	0	++++	++++
			–	–	–	R
OS	F.	Pyelitis, chronic; cystitis, acute	++	–	–	R
			–	–	–	R
			–	–	++	++
BD	F.	Pyelitis, chronic (pyelonephritis chronic†)	–	–	–	++++
			–	–	–	R
			–	0	–	R
			–	0	–	R
			++	0	++	++
			–	0	–	0
			–	–	++	++
LS	F.	Cystitis, chronic	+++	0	++	++++
CMu	F.	Cystitis, acute	–	–	–	R
			–	–	++++	++++
			–	–	+	++++
			++++	–	–	++++
			0	0	0	++++
AF	F.	Ureteral stricture; pyelitis, acute	++++	–	–	+++
BMcG	F.	Pyelonephritis, acute	++++	0	++++	++++
TS	F.	Not made	++++	0	++	++++
CV	F.	Not made	+	–	–	+++
EH	M.	No history	++	0	++	++++
CMi	F.	Pyelitis, chronic; cystitis, acute	–	–	–	0
			–	–	–	++++
			–	–	–	R
DS	F.	Pyelonephritis, chronic; cystitis, acute (pregnant)	–	–	–	++
FW	F.	Pyelitis, acute; cystitis, chronic	–	–	–	++++
MWa	F.	Pyelitis; cystitis, acute	–	–	–	++++

* S, susceptible *Bacillus coli;* F, susceptible *Bacillus dysenteriae* Flexner; H, *Bacillus coli* isolated from urine tested; R, resistant *Bacillus coli;* 0, no organism present.

† Started as mixed infection.

TABLE 1—*Continued*

CASE DESIGNATION	SEX		URINE LYTIC FOR			TYPE OF B. COLI PRESENT
			S	F	H*	
MWh	F.	Pyelitis; cystitis, acute	–	0	–	+++
BD	F.	Pyelitis, chronic	0	0	0	++
RA	M.	Pyelitis, acute; cystitis	0	0	0	++
EN	F.	Cystitis ?; urethral stricture acute exacerbation	–	–	–	R
			++++	++	–	R
GS	F.	Pyelitis; cystitis, chronic	–	–	–	R
			++	0	–	R
			–	0	–	R
			–	–	–	R
EF	M.	Pyelonephritis, acute (died)	–	–	–	R
			++++	0	–	R
ES	F.	Cystitis, chronic	++	–	–	R
HS	F.	Pyelitis; cystitis, chronic	–	–	–	R
			–	–	–	R
CR	F.	Pyelitis; cystitis, chronic	–	–	–	R
			–	–	–	R
HP	F.	Pyelitis; cystitis, chronic	–	–	–	R
LM	F.	Pyelitis; cystitis, chronic	–	–	–	R
AN	F.	Pyelitis; cystitis, chronic	–	–	–	R
			–	+	–	+
G	F.	Pyelitis; cystitis, chronic	–	–	–	R
			–	–	–	R
MP	F.	Pyelitis; cystitis, chronic	–	–	–	R
RN	M.	Pyelitis; cystitis, chronic	–	–	–	R
RH	M.	Pyelitis; cystitis, chronic	–	–	–	R
			–	–	–	R
			–	–	0	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
AB	F.	Pyelitis; cystitis, chronic	–	–	–	R
WD	M.	Pyelitis; cystitis, chronic	–	–	–	R
WMQ	M.	Pyelitis; cystitis, chronic	–	–	–	R
			–	–	–	R
			–	–	–	R
JA	M.	Cystitis, chronic; prostate, hypertrophy	++	–	–	R
			–	–	–	R
JF	M.	Cystitis, chronic; prostate, hypertrophy	–	–	–	R

TABLE 1—*Concluded*

CASE DESIGNATION	SEX		URINE LYTIC FOR			TYPE OF B. COLI PRESENT
			S	F	H*	
ED	M.	Cystitis, chronic; prostatitis	–	–	–	R
HM	M.	Cystitis; pyelitis; prostatitis, chronic	–	–	–	R
FY	M.	Cystitis, chronic; prostatitis, hypertrophy	–	–	–	R
MC	M.	Cystitis, chronic; prostatitis, hypertrophy	–	–	–	R
VS	F.	Pyelonephritis, acute	–	–	–	R
			–	–	0	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
			–	–	–	R
LH	F.	Pyelonephritis, chronic (died)	–	–	–	R
			–	–	–	R
AB	M.	Ureteral calculus; pyelitis, chronic	–	–	–	R
ES	F.	Carcinomatosis; cystitis, chronic	–	–	–	R
			–	–	–	R
DJ	F.	Pyelonephritis, acute and chronic	–	–	–	R
IR	F.	Pyelitis; cystitis, acute (Strep.)	–	–	–	++†
JS	M.	No diagnosis	++++	0	++	0
MC	F.		–	–	–	R
LBt	F.		–	–	0	R
RH	M.		–	–	–	R
GG	M.		–	–	–	R
ML	F.		–	–	–	R
WM	F.	No renal infection	–	–	–	R
BH	F.		–	–	–	R
LM	M.	Normal urine	–			
ML	M.		–			
P	F.		–			
W	F.		–			
HA	M.		–			
MC	M.		–			
D	M.		–			
AN	M.		–			
ER	M.		–			

‡ Coli contamination.

the number of specimens from a single patient. In the latter case, since positive findings may occur at times and negative results at others, it is necessary to adopt an arbitrary standard in order to classify the cases. This has been accomplished by including in the positive category all individuals whose urine contained the factors sought in any specimen of urine. Such variations as occur in the different specimens from the same patient will, however, receive consideration.

It is of some interest, however, to study the findings in urines without reference to the patients from whom they were obtained. Excluding the normal urines; that is the urines from individuals not known to have infection of the urinary tract 109 specimens are available for analysis. On the basis of the six conditions mentioned above, these urines may be distributed as follows:

	NUMBER	PER CENT
Bacteriophage and susceptible *Bacillus coli*	17	15.6
Resistant *Bacillus coli*	8	7.3
No *Bacillus coli*	1	0.9
Total	26	23.8
No bacteriophage and susceptible *Bacillus coli*	7	6.4
Resistant *Bacillus coli*	67	61.5
No *Bacillus coli* (normal urines)	9	8.3
Total	83	76.2
Susceptible *Bacillus coli*		24–25
Resistant *Bacillus coli*		75–75

Because of several facts among which are the meagerness of the material, and the variation in the number of specimens from different individuals, it is not safe to draw too many inferences from these data. It is, however, quite obvious that susceptible colon bacilli occur in infected urines, that the bacteriophage is likewise found in a fair proportion of the specimens, that bacteriophage is not found in uninfected urines, and that its presence may be coincident with either resistant[4] or susceptible bacteria. The single case in which bacteriophage was found in a urine in which no organism was demonstrated should not be regarded as an exception to the statement that bacteriophage is not found in uninfected urines, for this individual was undoubtedly infected although the organism had not been isolated.

It is the individual, i.e., the patient, however, in whom one is interested rather than in the urine, consequently an analysis on the basis of persons is far more significant than that based on urines. Adopting the suggestion previously made that the occurrence of bacteriophage or susceptible bacteria at any time in the urine from a single individual would place that person in the positive group with respect to the presence of these factors, the following analysis is presented:

	NUMBER OF INDIVIDUALS	PER CENT
Bacteriophage and susceptible *Bacillus coli*	13	25.0
Resistant *Bacillus coli*	5	9.6
No *Bacillus coli*	1*	1.9
Total	19	36.5
No bacteriophage and susceptible *Bacillus coli*	8	15.4
Resistant *Bacillus coli*	25	48.1
No *Bacillus coli*	0	0.0
Total	33	63.5
Susceptible *Bacillus coli*	21	41.0
Resistant *Bacillus coli*	30	59.0

* The case mentioned above which was certainly an infected individual.

In this study, the normal non-infected group of individuals is not included, since it was disposed of in the preceding table.

As in the preceding instance, the number of individual factors in the group is too small to permit of making strong assertions, yet it is evident that a susceptible bacterium occurs, at some time or at frequent intervals during an infection, in a fairly large percentage of the cases considered. The same statement applies to the bacteriophage. Neither bacteriophage nor susceptible bacteria are found consistently in all of the specimens from a single individual however. The possible significance of such a result will be discussed later.

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[4] This statement appears to be contradicted later. Let the word resistant here be understood as meaning not completely susceptible to dissolution. Also let susceptible be understood to mean not resistant. It will be seen that the above statement is not true for strains of *Bacillus coli* that are completely resistant or completely susceptible.

Not only did the specimens studied come from individuals; they came from males as well as females, and the cases represented both acute and chronic conditions of infection. Analyses of the results obtained in males as contrasted to those found in females as well as those found in chronic cases as distinguished from acute cases present some facts worthy of consideration. These analyses are contained in the following tables:

	MALES		FEMALES	
	Number	Per cent	Number	Per cent
Bacteriophage and susceptible *Bacillus coli*	1	5.9	11	30.5
Resistant *Bacillus coli*	3	17.6	3	8.5
No *Bacillus coli*	1	5.9	0	0.0
Total	5	29.4	14	39.0
No bacteriophage and susceptible *Bacillus coli*	1	5.9	8	22.2
Resistant *Bacillus coli*	11	64.7	14	38.8
No *Bacillus coli*	0	0.0	0	0.0
Total	12	70.6	22	61.0
Susceptible *Bacillus coli*	2	12.5	19	52.8
Resistant *Bacillus coli*	14	87.5	17	47.2
Total	16			

	ACUTE		CHRONIC	
	Number	Per cent	Number	Per cent
Bacteriophage and susceptible *Bacillus coli*	7	38.8	2	7.1
Resistant *Bacillus coli*	2	11.8	3	10.7
No *Bacillus coli*	0	0.0	0	0.0
Total	9	50.0	5	17.8
No bacteriophage and susceptible *Bacillus coli*	8	44.4	3	10.7
Resistant *Bacillus coli*	1	5.6	20	71.5
No *Bacillus coli*	0	0.0	0	0.0
Total	9	50.0	23	82.2
Susceptible *Bacillus coli*	15	83.3	5	17.9
Resistant *Bacillus coli*	3	16.7	23	82.1
Total	18	100.0	28	100.0

It is obvious that the incidence of bacteriophage and of susceptible colon bacilli is greater in females than in males, but it is also apparent that in acute conditions of infection the same factors are present to a much greater extent than in chronic states. The male patients who provided the material for this study were almost without exception subject to a chronic infection. Such a condition is generally true. Male patients suffering with urinary infection have at the time they undergo treatment, chronic infections. They are less often seen by the clinician when their infections are in the acute stages than are the females. On this account it can not be argued from the results presented here that males are less subject to infection with susceptible bacteria than are females nor that they less frequently harbor a race of bacteriophage in the infected urine.

DISCUSSION

In the introductory paragraph it was stated that the investigation might contribute to a better understanding of the source occurrence, permanence and fate of the bacteriophage in the urine and also that information of value in the bacteriophage therapy of urinary infections might be provided. These questions will now be considered from the standpoint of the data reported.

With regard to the occurrence of bacteriophage in the urine, but little is to be found in the literature. We are not concerned here with the appearance of the principle when artificially introduced into the body as after injection, but with its natural occurrence. Almost the only reference to this question is made by d'Herelle (1922) who found it in the urine. He does not state, however, whether this urine contained bacteria but claims that the principle was able to pass through the intestinal mucosa into the blood and thence to the urine through the kidneys. Such a sequence of events may occur, but only when there is an infection in the urinary tract; in spite of the comparatively small number of normal urines studied in this series, it seems safe to state that the normal, uninfected urine never contains the principle naturally. When injected into the circulation bacteriophage may be detected in the normal urine but except under such artificial conditions it is not likely to be found. Nevertheless, when an infection is present in the urinary tract the bacteriophage is detectable in many urines and further, it is found in nearly all specimens when the infection is acute. What then can be the source of this bacteriophage?

Before attempting the answer to the above question, it is necessary to consider the occurrence of the two types of *Bacillus coli* found. It is not conceivable that a susceptible bacterium could maintain its existence in a fluid medium in the presence of the bacteriophage. It would be dissolved. On the other hand it is well known that a type of bacterium exists which acts as a carrier of the bacteriophage. Such an organism has neither complete susceptibility nor complete resistance. It will be seen, however, that in a considerable percentage of the urines studied bacteriophage and organisms apparently capable of being dissolved were present together. These bacteria, then, being of the carrier or lysogenic type were responsible for the presence of the bacteriophage in the urine. They introduced and maintained the principle in the bladder and kidneys. In a subsequent study dealing with animal experiments and the action of bacteriophage in the body it will be shown that bacteriophage does not remain in the bladder except when a carrier or lysogenic type of bacterium is present.

With regard to the permanence of the principle in the urine, an examination of the first table shows that it seldom endures throughout the course of the infection. The bacteriophage appears and disappears. The disappearance is quite explainable; the lysogenic state being instable, is capable of change to a state of absolute susceptibility or resistance. When either of these conditions is reached, the bacteriophage disappears. Its reappearance can only be accounted for by reinfection with a lysogenic bacterium.

The fate of the principle depends upon the outcome of the struggle between the bacteriophage and the bacterium. If the bacterium is able to oppose an increasing resistance to the dissolving action of the principle, the time arrives when it becomes impossible to demonstrate bacteriophagy, either because the bacteriophage is no longer present, having been eliminated mechanically since there was nothing permitting its reproduction, or, as is more likely, because it has lost its virulence through association with a resistant bacterium. On the other hand, if the bacterium becomes less resistant, or if the bacteriophage gains in virulence, the bacterium is dissolved, and the bacteriophage, no longer having anything to retain it, is mechanically removed.

The results of the studies of the acute cases lend some support to these suppositions, since here we find bacteriophage and moderately susceptible bacteria. The condition cannot remain static. The struggle between the bacteriophage and the bacterium goes on, and, according to whether the bacterium or the bacteriophage gains the ascendency, the case becomes chronic or recovers. Might not this fact account for a number of spontaneous cures of pyelitis and cystitis?

Beyond a doubt there are other factors which play a rôle and influence the course of events, for we are not dealing solely with the relationship between the bacteriophage and the bacterium. The presence of tissue, bladder and kidney, and the presence of urine contributes something to the reaction—the possible modifying factors which will be considered later.

CASES TREATED

From time to time during the prosecution of this investigation opportunities for the treatment of cases of pyelitis and cystitis presented themselves. Inasmuch as the work already completed had furnished ample justification for undertaking therapeutic experiments, and since the reports of cases to be found in the literature provided not only further justification but descriptions of methods, the opportunities were accepted. It will be seen from a consideration of the cases presented that various means of administration of the bacteriophage were adopted. The procedures were suggested by the published records of treatments undertaken by Bazy (1925), Zdansky (1925), Courcoux (1922), and Alphonsi (1924). These cases are not presented with any claim as to the efficacy of the principle administered, nevertheless, they add to the number of instances of this nature in which bacteriophage therapy has been employed, and, regardless of the interpretation which one may choose to make of the results, it is obvious that improvement in the condition of the patients followed the administration of bacteriophage. Such a statement is not intended, however, to imply cause and effect.

Case 1. L. B., female, aged thirty-three years. This patient was referred from the Dispensary with a diagnosis of pregnancy complicated by pyelitis. After a cystoscopic examination on October 4, 1923, the following notes were made: Patient seven and one-half months pregnant complaining of frequency of urination; loss of weight; localized dull pain in right abdomen; burning and smarting associated with urination. The examination itself revealed the following conditions: Generalized cystitis; pus and bacilli in the right kidney pelvis; left kidney, normal. A kidney lavage of 2 per cent boric acid was given and the patient was asked to return for examination later. On October 9, the condition was unchanged and the treatment was repeated. Subsequent treatments with little or no improvement were continued until December 7 when delivery occurred.

Eleven days after delivery, examination showed that the condition of the bladder had changed but little. The left kidney had, however, become infected by this time. Cultures from the right and left kidneys and bladder were positive for *Bacillus coli.* The organism isolated proved to be susceptible to dissolution by a stock filtrate containing bacteriophage. On December 18, 5 cc. of bacteriophage were introduced into *each* kidney pelvis. The patient was discharged the next day and cautioned to report any return of symptoms. On January 17, she returned to the Dispensary and reported complete freedom from urinary troubles.

During the past two years she has been seen at intervals of five or six weeks because of conditions not associated with the genito-urinary tract. During this period she has had no return of her urinary troubles.

Case 2. M. W., female child, aged eight months. This child was admitted on October 26, 1924, to the Pediatric service. Her previous history was negative. Four days prior to admission, the mother noticed the first signs of sickness. These early symptoms, which consisted of vomiting and restlessness, persisted and were present on admission, when further observation indicated a dry and protruding tongue and dry lips. The urine was found to contain many pus cells and bacteria which proved to be *Bacillus coli.* A diagnosis of pyelitis was made as a result of these findings.

The treatment consisted of administration of large quantities of fluid, mostly by subcutaneous inoculation and nasal drips. Sodium bicarbonate, saline and glucose were given. These measures apparently producing little effect, a blood transfusion was given which was followed by a drop in temperature to 37.5 degrees, lasting for 24 hours. During this time, however, the patient's condition was not good. She continued to be apathetic and restless.

On October 30, the patient having grown progressively worse, and a blood culture having shown the presence of *Bacillus coli,* bacteriophage treatment was instituted. The filtrate used had not been shown to be active for the *Bacillus coli* causing the infection, nevertheless, 10 cc. of bacteriophage were introduced into the bladder by catheter at 5:30 p.m. An immediate drop in the temperature was observed and the patient improved clinically so that by the next morning a very noticeable change for the better was observed.

In the twenty-four hours succeeding the first treatment a race of bacteriophage had been found which was capable of producing lysis of the colon bacillus in question. Consequently the treatment was repeated on October 31, following which defervescence occurred. The patient continued to improve and was discharged a week later as cured. The temperature record is presented here.

The laboratory study of this case was not as complete as could be desired, yet studies of the urine after the treatment revealed a remarkable increase in the number of pus cells and a clumping of the bacteria with loss of motility. The pus persisted for several days and gradually disappeared. The bacteria likewise could be seen in smears for several days, remaining clumped and non-motile. They gradually decreased in numbers, although at the time the patient was discharged the urine still contained bacteria. The blood leukocyte count increased from 22,000, which it was upon admission, to 40,000 shortly after the administration of the bacteriophage. It had not returned to normal upon the discharge of the patient.

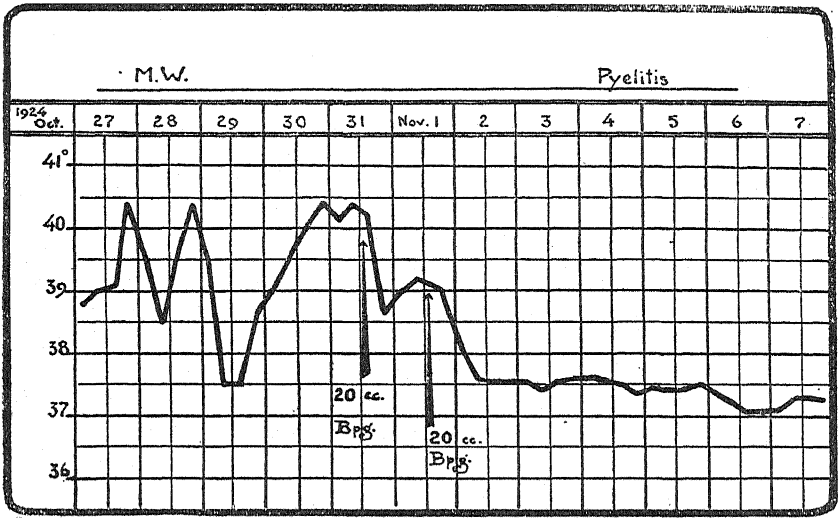

Fig. 1

Two weeks after leaving the hospital the child was brought in for examination. At that time it was apparently well. The urine was free of bacteria. About nine months after the patient's discharge, the mother returned for the purpose of reporting that the child had been in excellent health since leaving the hospital.

Case 3. E. R., female, aged thirty. This patient was admitted to the hospital on April 9, 1925, with a diagnosis of pyelitis complicating pregnancy. She was in the seventh month of her pregnancy and reported an attack of severe pain in the right lower quadrant. This pain was first noticed five days prior to admission and was persistent, being associated with chills and fever. An examination of the urine showed pus cells and many bacteria which were motile and not clumped.

A colon bacillus susceptible to lysis with a stock filtrate containing bacteriophage was cultured from the urine. On april 12, three days after admission, the patient was given a subcutaneous inoculation of 2 cc. of this bacteriophage. A slight rise in temperature followed, but within twelve hours the temperature had fallen, and it continued to drop, reaching 37°C. twenty-four hours after the first inoculation. At this time a second inoculation of bacteriophage (2 cc.) was given which was in turn followed by a rise in temperature amounting to one degree. The temperature quickly dropped to normal, however, and from this time until the discharge of the patient, six days later, it remained so. The pain which had been persistent, relieved only by posture, disappeared shortly after the treatment was begun and did not reappear. The patient was discharged a week after the first treatment. She was delivered on July 1, at which time she had no urinary symptoms and

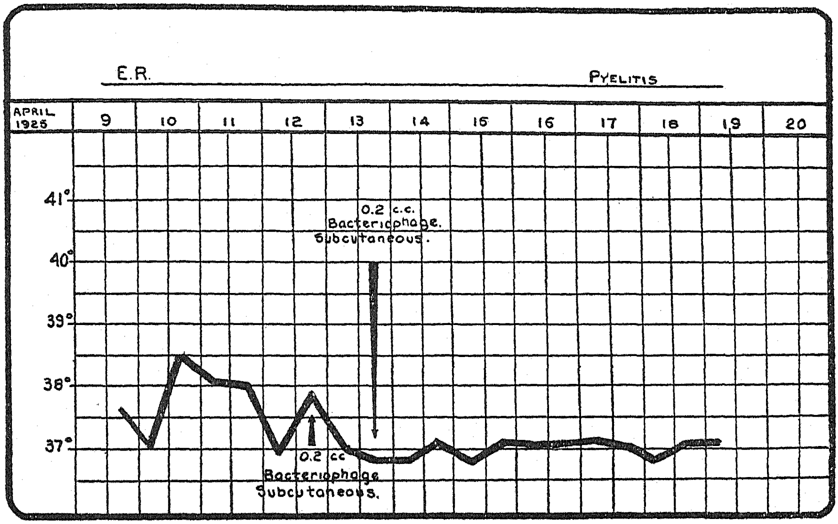

FIG. 2

the urine was free of bacteria nor had she had urinary symptoms in the interval.

The laboratory findings in this case were as follows: There was no change in the leukocytic count following inoculation of bacteriophage. The urine, however, contained large numbers of pus cells which increased after administration of the bacteriophage and then gradually decreased. The bacteria presented the same phenomenon observed in the preceding case. Clumping with loss of motility was marked. At the time of discharge the urine still showed an occasional bacillus, but in July when the patient came in for delivery there were no bacteria in the urine. The temperature record is presented as it shows a sharp drop following inoculation of the bacteriophage.

Case 4. A. R., female, aged fourteen months. In this case the urinary infection followed a protracted, intermittent diarrhea and, at the time of admission, the child was in a decidedly malnourished condition having a thrush infection and a bilateral otitis media in addition to the pyelitis. There was also a question of respiratory infection. On account of the many complications, the temperature record in this case is of little value in indicating the effects of bacteriophage therapy for the urinary infection. The urine contained many pus cells and large numbers of motile Gram-negative bacilli which on culture proved to be *Bacillus coli.* When subjected to *in vitro* tests the bacillus was dissolved by the race of bacteriophage used in the treatment of the previous cases. A specimen of urine obtained by catheterization at the time of the second treatment showed many clumped, non-motile bacilli and many pus cells. Forty-eight hours after the second treatment a catheterized specimen of urine contained an occasional bacillus, non-motile, and very few pus cells. Two days later, the urine was sterile. No bacilli were seen in smears and no growth occurred in cultures of the urine. No pus cells were present. The patient remained in the hospital for several weeks during which time no bacteria were found in repeated specimens of urine, and at the time of discharge the urine was sterile. No further discussion of these cases will be presented since it is the opinion of the writer that interpretation of the results of treatments of so few cases is unwarranted.

SUMMARY

Routine studies of urines from patients having urinary infections revealed the fact that bacteriophage was present in about 25 per cent of the urines while *Bacillus coli* susceptible to the action of bacteriophage was present in the same proportion of the specimens. The urines in which the susceptible colon bacilli were found were not necessarily the same as those in which bacteriophage was demonstrated. Normal urines, that is, urines not known to contain bacteria, were found to be free of bacteriophage.

When individual cases rather than urines were considered it was found that over 36 per cent of the cases studied had bacteriophage in one or more of the specimens of urine examined, while over 40 per cent (not necessarily including the above 36 per cent), were infected with a colon bacillus capable of being dissolved by a race of bacteriophage.

Almost without exception, the chronic cases provided urines in which only resistant bacteria were found, while the acute were seldom due to this type of colon bacillus. Bacteriophage too was found exclusively in the urines from individuals having acute infections.

The incidence of bacteriophage and susceptible colon bacilli in males and females was affected by the above condition. In practically every instance the males were suffering with chronic infections. Consequently the males, except in one case, were never a source of bacteriophage or susceptible bacteria.

Four patients subjected to treatment with the bacteriophage showed definite improvement after the treatment.

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BACTERIOPHAGY IN URINARY INFECTION[1]

PART II. BACTERIOPHAGY IN THE BLADDER

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Bacteriophage therapy of many infections, occasioned by various organisms and occurring in different tissues, has been attempted with a fair degree of success. Numerous publications bearing upon the treatment of dysentery, typhoid and paratyphoid fevers, urinary infections and plague, and of localized infections caused by the staphylococcus, streptococcus and other organisms have appeared in the literature within the past three years. Nearly all of these communications testify to the success of this method of treatment, but for the most part the work has been empirical. Reasoning that a substance capable of bringing about the complete dissolution of a given bacterium when inoculated into a broth culture or suspension of the organism in a test tube should bring about the cure of an infection because of a similar occurrence in the body of an infected individual, the chief concern of a majority of these investigators has been to bring the bacteriophage into contact with the bacterium. However striking the results obtained by such a method may be, until the mechanism of the reaction is better understood, and until methods based upon demonstrated facts have been devised, it cannot come into general acceptance as a scientific procedure.

The problems confronting the investigator bent upon determining the behavior of the bacteriophage in the body depend to a considerable extent upon the location and nature of the infection. It is perfectly obvious that each infection and each tissue infected presents a different problem with respect to the action of the bacteriophage. Hence, it is not logical to infer that because bacterial dissolution occurs in the test tube in the presence of bacteriophage, it will likewise take place in a given tissue. That bacteriophage is extremely susceptible to changes in the environment has been shown by numerous investigators.[2] Consequently, within the tissues or in contact with various body fluids, it must be modified to an extent impossible to predict. Not only may the bacteriophage be modified by various physical and chemical conditions, but such physiological factors as the leucocytes as shown by d'Herelle and Eliava (d'Herelle, 1921), may become stimulated in the presence of the principle. Because of these facts it is evident that to administer bacteriophage as a therapeutic agent in infectious diseases without a knowledge of the limitations imposed by the environment is to eliminate the possibility of determing its rôle as a factor in the result.

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[1] This paper is taken from a dissertation presented for the degree of Doctor of Philosophy in the Department of Pathology and Bacteriology, Yale University.

So long as investigators interested in the therapeutic application of the bacteriophage confine their efforts to a study of the material they are using and its effects upon the bacterium *in vitro* and neglect the modifications which bacteriophagy may undergo *in vivo,* there will be little progress in the use of bacteriophage as a therapeutic agent. The realization of this fact was responsible for the work set forth in the following pages. *Bacillus coli* infections of the urinary tract offer unusual opportunities for the study of bacteriophagy within the body. The location and character of the lesions, the variations in the bacterium, and the environmental conditions provided by the secretion of urine and of mucus provide almost limitless material for investigation. Consequently, infections of this character were selected for study.

Having found in a previous investigation[3] that normal urine; that is, urine not known to contain bacteria, was free of bacteriophage, while this principle occurred in 25 per cent of urines derived from infected bladders or kidneys, and that colon bacilli susceptible to lysis occurred in a like percentage of cases, although not necessarily in the same cases, the question arose as to the source of the bacteriophage and the reason for the existence of the different types of *Bacillus coli*. Other questions developed in the course of the investigation such as; the effect upon the bacteria of bacteriophage introduced into the bladder; the reason for the absence of bacteriophage in normal urines; the explanation of the infectiousness of the colon bacillus; and the effects of the presence of urine and of mucus upon the bacterium and upon the bacteriophage. The attempts to answer these questions through experiments and the results obtained are described in the following pages.

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[2] Gratia (1921), Eliava and Pozerski (1921), Scheiddiger (1923), Davison (1922.) and Reichert (1924). Hydrogen ion concentration.

Da Costa Cruz (1923a and b). Electrolytes.

Doerr (1922), Doerr and Berger (1922), Nakumura (1923a and b), Hauduroy (1923). Viscosity of medium.

Hauduroy (1925). Bile.

[3] Bacteriophagy in urinary infection. Part I (in press).

EXPERIMENTAL WORK

As to the source of the bacteriophage found in many infected urines it is evident that it is in some manner connected with the presence of bacteria since it is never present in normal urine. In addition to the eleven human urines reported in a previous paper, examinations of the urines of about twenty-five normal rabbits, five guinea-pigs, and three dogs have given the same result. Since the presence of bacteria was always associated with the presence of bacteriophage, attempts were made to infect rabbits by introducing colon bacilli into the bladder. The outcome of these efforts is shown in table 1.

Although not providing conclusive evidence, the above results tend to indicate the so-called lysogenic strains of *Bacillus coli* as responsible for urinary infection. By lysogenic strains is meant, strains capable of carrying the bacteriophage, that principle existing in symbiosis with the bacterium and subject to all of the laws which govern such a condition. Infection with such strains results in the appearance of bacteriophage in the infected region. Such a result has been demonstrated repeatedly by other investigators and needs no further consideration here. One must, however, eliminate the other possibilities in order to show that the lysogenic bacterium is alone responsible for the presence of bacteriophage in the urine. This has been done to a considerable extent in the present investigation.

To indict the *lysogenic* strains of *Bacillus coli* as responsible for the urinary infections caused by this bacterium, is a matter of some significance. The longer the duration of symbiosis, the greater becomes the resistance of the bacterium to dissolution by any bacteriophage (d'Herelle, 1926), consequently, it is not to be

TABLE 1

RABBIT	SUSPENSION INTRODUCED INTO BLADDER*	DATE	RESULT
I	10 cc. Coli S†	5/10/24	Urine sterile 8/11/24; no bacteriophage
II	5 cc. Coli S	5/ 9/24	Urine contained Coli S 7/12/24; no bacteriophage
III	5 cc. Coli S	7/ 9/24	Urine contained Coli S 7/21/24; no bacteriophage. Urine sterile 8/11/24; no bacteriophage
IV	5 cc. Coli S	7/12/24	Urine contained Coli S 7/21/24; no bacteriophage Urine sterile 8/11/24; no bacteriophage
V	2.5 cc. Coli S (intravenously)	7/12/24	Urine contained Coli S 7/18/24; no bacteriophage. Died 7/23/24, Abscesses in kidneys and bladder. No bacteriophage
VI	1 cc. Coli S	7/ 6/24	Urine contained Coli S 7/7/24 and 8/6/24; no bacteriophage
VII	10 cc. Coli R‡	5/10/24	Urine sterile 8/11/24
VIII	10 cc. Coli R	10/21/24	Urine contained Coli R 10/29/24; no bacteriophage. Urine sterile 11/4/24; no bacteriophage
IX, X, XI, XII	5 cc. Coli R	8/19/24	Urine sterile 9/9/24; no bacteriophage
XIII	20 cc. Coli S	10/10/25	Urine sterile 10/12/25; no bacteriophage
XIV	20 cc. Coli R	10/12/25	Urine sterile 10/15/25; no bacteriophage

* A suspension prepared by washing an eighteen-hour agar slant growth with 5 cc. of broth. Introduced into bladder by catheter.

† Colon bacillus capable of being dissolved by bacteriophage, race 3620 in six hours.

‡ Colon bacillus not visibly affected by bacteriophage.

TABLE 2

RABBIT	STATE OF BLADDER*	PROCEDURE	RESULTS
I	Normal	1 cc. bacteriophage intravesicularly	Disappeared twenty-four hours later
II	Normal	5 cc. bacteriophage intravesicularly	Disappeared twenty-four hours later
III	Normal	10 cc. bacteriophage intravesicularly	Disappeared twenty-four hours later
IV	Normal	2 cc. bacteriophage intravenously	Appeared in urine in twelve minutes; disappeared in one and a half hours
V	Normal	2 cc. bacteriophage intravenously	Same as above
VI	Normal	10 cc. bacteriophage + 20 cc. Coli S intravenously	Bacteriophage and bacteria present after twenty-four hours; disappeared after forty-eight hours
VII	Normal	10 cc. bacteriophage + 25 cc. Coli S intravenously	Same as above
VIII	Normal	10 cc. bacteriophage + 20 cc. Coli R	Bacteriophage disappeared after twenty-four hours; bacteria disappeared on third day†
IX	Contained Coli R. Present for for 6 days	10 cc. bacteriophage intravesicularly	Bacteriophage disappeared after twenty-four hours; bacteria disappeared after five days†
X	Had contained Coli S for 2 months	10 cc. bacteriophage intravesicularly	Bacteria and bacteriophage absent 3 days later

* Urines were alkaline pH 7.6–7.8.

† The disappearance of the bacteria occurs within this time in the absence of bacteriophage.

expected that bacteriophage can be used successfully in cases of long standing. This statement assumes, what has yet to be demonstrated, that dissolution of the bacterium is alone responsible for any beneficial results from the therapeutic use of bacteriophage. We will return to this question later.

It is interesting to note the difficulty of producing *Bacillus coli* infections in rabbits. In practically every instance the bacteria could not be demonstrated in the urine after forty-eight hours. Helmholz and Beeler (1918) were able to produce pyelitis in about 50 per cent of rabbits by introducing colon bacilli into the bladder. These investigators were working with strains isolated from rabbits having a naturally acquired infection, however. It may be that such strains have a greater virulence for rabbits than do strains isolated from urines from human pyelitis cases.

The work so far described is concerned primarily with the influence of bacteria upon the appearance of bacteriophage in the urine. There is, however, interest in another phase of the same problem. Disregarding the bacteriophage already present, what is the fate of bacteriophage artificially introduced into the bladder? As a basis for the consideration of this problem, table 2 is presented.

That bacteriophage is rapidly eliminated from the bladder is shown by the above table. The presence of susceptible organisms slightly increases the persistence of the principle, but in any event its residence in the bladder is brief. Apparently it made no difference whether the colon bacilli had been in the bladder for some time previous to the bacteriophage instillation or whether bacteria and bacteriophage were introduced simultaneously. Obviously, this result fortifies the hypothesis advanced above that the presence of bacteriophage is due to an infection with the "lysogenic type" of colon bacilli, for we know that in infection of man, bacteriophage can be demonstrated repeatedly in certain cases.

The results reported in table 2 have been confirmed in a number of instances in treatment of pyelitis and cystitis in man. No protocols are given here since the cases have already been described,[4] but it was always true in the cases studied that bacteriophage introduced into the human bladder was eliminated within twenty-four to forty-eight hours. This fact has its application to therapy, for in those cases where disappearance of the organisms did not occur within forty-eight hours after intravesicular instillations of bacteriophage it indicates that their subsequent disappearance could not be attributed to *dissolution* of the bacteria. If the principle is at all concerned with the ultimate reduction of the infection it must have operated in some manner other than dissolution.

The observations so far reported have been concerned with the presence or absence of bacteriophage in the urine. It has been suggested, however, that given a susceptible colon bacillus and an active bacteriophage in the urinary tract, the phenomenon of lysis might be considerably modified as compared to the manifestations of bacteriophagy *in vitro*. Indeed, the only direct evidence that bacteriophagy occurs at all in the bladder is furnished by Marcuse (1924), who infected guinea pigs by introducing colon bacilli into the bladder and observed the disappearance of the organisms after bacteriophage was instilled. The colon bacilli disappeared in the treated animals but remained in the controls. The disappearance of colon bacilli following introduction of bacteriophage has been reported by many investigators notably Hauduroy (1924), Philibert (1923–24), and d'Herelle (1926), treating human cases, and, in the present investigation, when rabbits were treated. In such observations, however, the only control was the previous condition in the bladder. The work of Marcuse is fairly conclusive so far as the disappearance of the organisms is concerned, but it does not exclude the possible intervention of other factors activated by the bacteriophage. Such a problem is exceedingly difficult but is of extreme importance. Should it prove that recovery from infection subsequent to bacteriophage introduction was dependent upon some factor other than dissolution, such as the intervention of leukocytes, urine, mucus, or some other substance the methods, as well as the concepts, of therapy would be materially modified.

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[4] Bacteriophagy in urinary infection. Part I.

The work to be reported in the following pages represents an attempt to supply the answer to these questions. That it affords no basis for definite conclusions is admitted, yet it does indicate the possibilities and establishes certain points.

It is first necessary to determine the possibility of bacterial dissolution within the bladder cavity, and to observe whether the rate of lysis is influenced by the site of the reaction, whether the dissolution, if it occurs, is accompanied by any temperature changes in the animal, and what if any differences exist in the appearance of the colon bacilli during dissolution *in vivo* and *in vitro* as determined by examination of smears.

Since it had been found that it was difficult if not impossible to infect rabbits by introducing *Bacilli coli* into the bladder, the experiments were accomplished by instillation of 20 to 25 cc. of a suspension of the bacilli by catheter. Two to 10 cc. of bacteriophage were instilled immediately after the bacilli. The catheter was retained, closed by means of a pinch cock, and opened at intervals to remove specimens. A control animal received the same treatment except that no bacteriophage was introduced. The progress and degree of lysis was determined by comparison of the turbidity of the samples removed simultaneously from each rabbit as well as by examination of smears in which counts of the number of bacilli per field provided an index of the degree of dissolution. The study of these smears likewise provided other data which will be considered later. The results of these experiments appear in the protocols in table 3.

It is noticeable in all of these experiments that there occurred a decrease in the number of bacilli per field in smears of specimens taken from the bladder. This decrease followed an initial increase which reached its maximum between the second and the third hours. This fact admits of two explanations. Either the decrease was apparent only, being due to the dilution caused by the continued secretion of urine, or it was real, due to the operation of some agent other than the bacteriophage, operative in the control as well as in the test animals. The evidence afforded by the experiment in which a test tube control was used seemed to indicate that dissolution was more rapid in the rabbit, for the

TABLE 3

TIME	APPEARANCE OF URINE†		BACTERIAL COUNT PER FIELD		TEMPERATURE‡ °F.	
	I	II	I	II	I	II
Experiment I. *B. coli* susceptible						
p.m.					*°F.*	*°F.*
3.00*	Clear	Clear	0	0	99.8	99.6
3.50	Cloudy	Cloudy	4	4	98.4	98.6
4.15	Cloudy	Cloudy	4	4	98.2	98.2
4.45	Cloudy	Cloudy	27	15	98.4	98.6
5.00	Cloudy	Cloudy	25	20	97.2	98.8
5.20	Turbid	Turbid	40	30	97.2	98.4
6.00	Turbid	Clearing	50	30	97.2	98.4
6.20	Turbid	Clear	40	0	97.2	97.2
Experiment II. *B. coli* susceptible						
p.m.						
12.00*	Clear	Clear	0	0	99.0	97.8
12.45	Turbid	Turbid	22	20	98.4	97.5
1.15	Turbid	Turbid	20	15	98.8	96.4
2.15	Turbid	Turbid	30	25	98.7	96.3
2.45	Turbid	Turbid	15	10	98.6	96.3
3.15	Turbid	Clear	6	3	98.8	95.2
3.45	Turbid	Clear	5	0	99.0	96.2
4.15	Turbid	Clear	2	0	99.4	96.7
Experiment III. *B. coli* resistant						
p.m.						
3.45*	Clear	Clear	0	0	99.1	100.5
4.45	Turbid	Turbid	10	10	99.3	98.8
5.45	Turbid	Turbid	30	12	99.6	99.8
6.30	Turbid	Turbid	30	20	99.7	99.3
7.30	Turbid	Turbid	30	30	99.7	99.4
Experiment IV. *B. coli* resistant						
p.m.						
12.00*	Clear	Clear	0	0	95.9	97.3
1.00	Turbid	Turbid	4	8	93.6	96.0
2.00	Turbid	Turbid	6	20	93.6	95.0
3.00	Turbid	Turbid	18	50	92.0	95.4
4.00	Turbid	Turbid	30	50	92.3	97.1
5.00	Turbid	Turbid	10	25	92.0	97.7
6.00	Turbid	Turbid	10	30	92.8	98.8
7.00	Turbid	Turbid	12	20	92.5	99.2
8.00	Turbid	Turbid	2	20		
a.m.						
10.00	Clear	Clear	0	0		

TABLE 3—*Continued*

TIME	APPEARANCE OF URINE†		BACTERIAL COUNT PER FIELD		TEMPERATURE‡ °F.	
	I	II	I	II	I	II
Experiment V.§ *B. coli* susceptible						
a.m.					*°F.*	*°F.*
10.55*	Clear	Clear	0	0	99.9	98.6
11.30	Turbid	Turbid	10	15	98.5	98.6
12.00	Turbid	Turbid	15	30	97.8	98.6
p.m.						
1.00	Turbid	Turbid	60	60	98.3	98.6
1.45	Turbid	Turbid	15	70	98.3	98.6
2.15	Clearing	Turbid	3	50	98.8	98.6
2.45	Clear	Turbid	0	40	99.0	98.6
a.m.						
8.00		Clear		2		98.6

* Animals catheterized.

† In experiment I, I is control animal, II is test animal. In experiment V, II is control tube.

‡ Temperatures were taken every 15 minutes but showed no great variations.

§ Instead of a control animal in this experiment a control tube was used and bacteriophage added. The amounts of culture and bacteriophage being the same as in the rabbit.

striking difference in the time required for complete clearing of the medium—less than four hours in the bladder and nearly twenty-four hours in the test tube—could hardly logically be attributed to dilution. As further indication of the operation of other factors in the bladder, the time required for the elimination of all bacteria, whether susceptible or resistant and whether bacteriophage was present or absent, was so brief that dilution alone could scarcely account for the occurrence.

When the factor of dilution, and in fact the presence of urine in the bladder was removed by ligation of both ureters of rabbits, lysis proceeded at the same rate in the bladder and in the test tube. In the control animal no diminution in the number of organisms occurred during two and one-fourth hours. Since, however, this period was less than the time usually required for reduction in the number of organisms, the experiment is far from conclusive. Owing to the extreme urgency exhibited by rabbits so shortly after the operation necessary for placing the ligatures, and to the consequent impossibility of introducing sufficient fluid to continue the experiment over a longer period, other experiments were devised which will be described later.

The above experiments do, however, show that bacteriophage can effect dissolution of bacteria in the bladder and confirm to this extent the results of Marcuse. They further show that no significant temperature reaction accompanies dissolution. The examination of the smears beside yielding the information as to the progress of dissolution afforded some other interesting data which will be considered later.

To return to the question of the effects of factors other than the bacteriophage upon colon bacilli in the bladder and to the effects of factors present upon the bacteriophage and upon bacteriophagy, it has been seen that ligation of the ureters did not provide a satisfactory experiment. Removal of the bladder would accomplish the elimination of the urine as a factor, but would also eliminate any substances supplied by the blood.

Any substances affecting either the bacteria or the bacteriophage within the bladder must have their origin in the bladder tissue itself, in the blood supplying the bladder, in the urine, in the mucus present in the urine, or in the products of bacterial metabolism. As a source of such substances, the blood can be eliminated, for not only is blood excluded fron direct contact with the bacteria or bacteriophage, but such elements as can leave the blood vessels, red blood cells and leukocytes, never appeared in the specimens examined in preceding experiments. Thus by removal of the bladder, the effect of bladder tissue itself—either alive or dead—and the effects of bacterial metabolism can be studied. The urine and the mucus can be investigated independently.

In table 4 are given the results obtained by introducing bacteriophage and colon bacilli into bladders removed from rabbits and suspended in Ringer-Locke solution, oxygenated, and kept at 37°C.

Apparently living bladder tissue has little or no effect upon bacteriophagy, lysis occurring in the same interval in the control tube as in the bladder. On the other hand, dead tissue not only increases the rate of the reaction, but when added to a broth culture is able to bring about a degree of dissolution, although no bacteriophage has been added. The presence of urine actually retards bacteriophagy to a marked degree.

TABLE 4

BLADDER	BLADDER CONTAINED				AMOUNT OF LYSIS AFTER HOUR:				
	Broth	Urine*	B. coli S	B/P 3620	First	Second	Third	Fourth	Twenty-fourth
	cc.	*cc.*	*cc.*	*cc.*					
I	25	0	2	1	0	0	+++	++++	++++
II	0	25	2	1	0	0	0	0	++++
III	25	0	5	1	0	++	–	–	++++
IV	0	25	5	1	0	0	–	–	++
V	25	0	5	1	0	++	++	++++	++++
TEST TUBE + BLADDER TISSUE									
I	25	0	2	1	0	0	+++	++++	++++
II	25	0	5	1	0	++++	–	–	++++
III	25	0	5	0	0	0	–	–	+
IV	25	0	5	1	0	+	++	++++	++++
V	25	0	5	0	0	0	+	+	++++
VI		25	5	1	0	0	0	0	++
TUBE	CONTENTS OF TUBE				AMOUNT OF LYSIS AFTER HOUR:				
	Broth	Urine	B. coli S	B/P 3620	First	Second	Third	Fourth	Twenty-fourth
	cc.	*cc.*	*cc.*	*cc.*					
I	25	0	2	1	0	0	+++	++++	++++
II	0	25	2	1	0	0	0	0	++++
III	25	0	2	0	0	0	0	0	0
IV	0	25	2	0	0	0	0	0	0
V	25	0	5	1	0	+	–	–	++++
VI	0	25	5	1	0	0	–	–	+

* Rabbit urine used.

Each of the latter two observations merits further investigation, the effect of urine in particular, since, although dead bladder tissue cannot play much of a rôle in the bacteriophage therapy of cystitis, urine is especially significant because it is the medium in which bacteriophagy, if it takes place, must occur. The question of the effect of urine is still under investigation.

Demonstrations of bacteriolytic substances in tissues have been frequent. McKinley (1923) found a principle in tissues, both normal and infected, which he proved to be bacteriophage. Fleming and Allison (1922) describe a bacteriolytic substance in tissues and in secretions such as tears. They claim that this principle is not a bacteriophage and call it "lysozyme." Petrovanu (1924) isolated a substance from dried and ground up intestines capable of dissolving certain cultures of Cholera vibrios. In the present investigation, the substance found to cause dissolution in one of the tubes (V, indicated in table 4) was capable of producing plaques, and was still active in a filtrate after five contacts with *Bacillus coli* and intervening filtrations, a fact which is much in favor of its being bacteriophage. Further, four bladders from guinea pigs gave a similar principle which was apparently not present in equal amounts and strength in the different bladders, for in all but one instance it was necessary to make several contacts of filtrates and colon bacilli before disclosing the principle. To prove conclusively that this principle is given off only by dead tissue is a difficult problem, for few means are available for keeping a tissue surviving while submitting it to treatment necessary to extract the bacteriolytic agent. Yet so long as the bladder tissue survived in the Ringer-Locke's solution used in these experiments, bacteriophage was not to be found. Thus, although the evidence is slight, there is some indication that living or surviving tissue retains bacteriophage which may be released upon the death of the tissue. Several other hypotheses are possible, but the application of the observations to the problem at hand is not hypothetical. It is quite evident that the living bladder tissue itself contributes little to the phenomenon of bacteriophagy *in vivo* at least to the extent of accelerating the process.

The work thus far described, however, leaves the question still unanswered as to what, if anything, causes more rapid lysis in the bladder than in the test tube. There remains only the mucus to be considered. The secretion of this substance is particularly abundant in rabbits.

In the examination of the smears prepared from specimens removed from the bladder in the living animal, a peculiar phenomenon was observed. The bacilli were at first clumped, giving the appearance of typhoid bacilli in a microscopic Widal test.

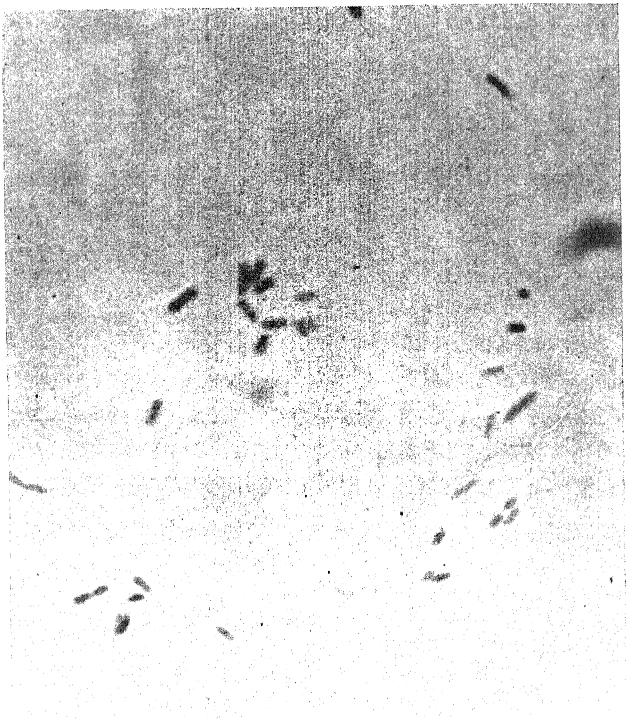

FIG. 1. × 1200

These clumped bacilli then became swollen and stained irregularly. The swelling was so great that the bacilli became from four to six times their normal dimensions. The two microphotographs presented here illustrate the change (figs. 1 and 2).

The first is of a smear prepared from a culture containing the same number of bacilli per cubic centimeter as were in the specimen from which the second smear was prepared. The colon bacilli shown in figure 1, however, were developing in a test tube, those shown in figure 2 were growing in the bladder of a rabbit. Bacteriophage was present in both cultures. Other smears showed the same phenomenon, however, in the absence of bacteriophage. Since making these observations, many instances of a similar occurrence in man have been observed. In many urines from cases of pyelitis and cystitis, the bacilli are clumped, lose motility, and tend to stain irregularly and to evidence more or less pleomorphism.

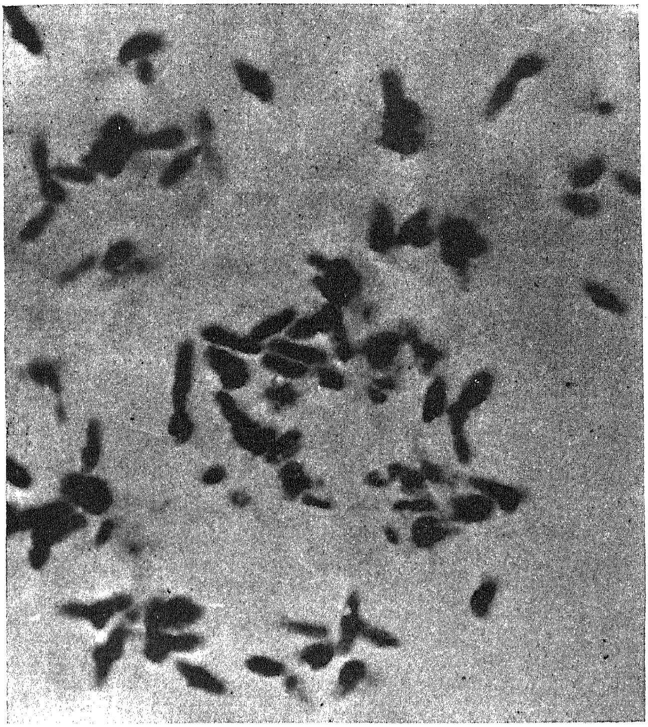

Fig. 2. × 1200

It has already been remarked that rabbit urine is particularly apt to contain large quantities of mucus, and it was in those urines in which mucus was most abundant that swelling and clumping were most marked. Some mucus obtained in a specimen of rabbit urine was washed several times with saline, and added to about 5 cc. of broth in two tubes. One was kept as a control of the sterility of the mucus, the other was inoculated with *Bacillus coli.* Both tubes were incubated at 37°C., the control remaining sterile. The colon bacilli from the other tube reproduced the phenomenon previously observed. Clumping and swelling became marked. Although there was no appreciable increase in the number of colon bacilli during several days of incubation, the organisms did not disappear but remained viable. Neither the inoculated tube nor the control contained demonstrable bacteriophage. It would appear from this observation that the mucus present in the bladder can exercise considerable influence upon bacteria invading the organ. Although it is not bactericidal or bacteriolytic, it has an inhibitory action which may be entirely mechanical. Through the agglutination which it causes it may aid in the normal process of removal of bacteria from the bladder. Certainly this clumping can account for a considerable proportion of the apparent decrease in organisms observed in smears. The question as to the effect of this mucus upon bacteriophage and upon bacteriophagy is at present under investigation.

CONCLUSIONS

1. Bacteriophage is not found in rabbits' urine when bacteria of any type except the lysogenic strains are put into the bladder and maintained there for varying periods of time.
2. The introduction of colon bacilli into the body by the enteral or intravenous route fails to cause bacteriophage to appear in the bladder.
3. Damage to the bladder wall by means of hydrochloric acid does not result in the appearance of bacteriophage in the urine.
4. When introduced into the bladder, bacteriophage is eliminated within twenty-four to forty-eight hours.

As a result of these findings it is suggested that infection with lysogenic strains of *Bacillus coli* is alone responsible for the existence of bacteriophage in the urine.

5. Lysis of colon bacilli through the action of the bacteriophage can take place in the bladder.
6. Urine exercises an inhibitive action upon bacteriophagy.
7. Mucus, although apparently not affecting bacteriophagy, acts upon the colon bacilli in such a manner as to promote their removal from the bladder.
8. Surviving bladder tissue has no effect upon bacteriophagy.
9. Dead bladder tissue releases a principle resembling bacteriophage.

While it is impossible, on the basis of these experiments to state through what agency and to what extent modifications occur, it is obvious that bacteriophagy is not the same in the bladder as it is in the test tube.

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GROWTH OF SPORE-FORMING ANAEROBES AT 50°C.

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The following observations were made during the course of some studies on anaerobes in sewages and sewage effluents. While the work was in progress a paper by Damon and Feirer (1925) on thermophilic anaerobes was published. These authors described four species which were isolated from horse manure and grew at 50° and 55°C. The authors were apparently unaware of a paper by Veillon (1922) in which he described three thermophilic anaerobes which developed both at 37° and 50°C. However, the organisms isolated by Damon and Feirer do not correspond with the descriptions of those studied by Veillon. None of these anaerobes appears to belong to the commonly known spore-forming types.

I have shown that the anaerobes most commonly found in sewages and effluents are *Cl. welchii* and *Cl. sporogenes* (Greer, 1925). On the sulphite-glucose-iron medium of Wilson and Blair (1924) these organisms produced black colonies when grown at 37°C. When the plates were incubated at 50°C. these same anaerobes appeared. The colonies were somewhat larger and developed more rapidly at the higher temperature.

Single cell strains of a number of spore-forming anaerobes were then studied. The organisms used were *Cl. aerofetidus*, *Cl. bifermentans*, *Cl. botulinum* A, *Cl. botulinum* B, *Cl. chauvei*, *Cl. edematiens*, *Cl. fallax*, *Cl. histolyticum*, *Cl. putrificum*, *Vibrion septique*, *Cl. sporogenes*, *Cl. tetani*, *Cl. tertium* and *Cl. welchii*. The cultures were made in beef heart medium, sealed with vaseline and incubated at 50°C. Observations of growth were made after four, eight and twelve hours and one, two, three, four and five days. All the above anaerobes except *Cl. histolyticum* and *Cl. tetani* showed growth, at least by the end of the fifth day. These two organisms had not developed at the end of ten days. Duplicate cultures incubated at 37°C. all showed growth in five days. In many cases growth was evident at 50°C. as soon as at 37°C. *Cl. sporogenes*, *Cl. welchii*, *Cl. fallax* and *Cl. tertium* produced gas earlier at 50°C. than at 37°C.

Cultures of *Cl. welchii* and *Cl. sporogenes* were made in milk and coagulated egg medium, respectively, to determine whether or not these characteristic reactions occurred at the higher temperature. Both the stormy fermentation of milk by *Cl. welchii* and the liquefaction of coagulated egg white by *Cl. sporogenes* took place as rapidly at 50°C. as at 37°C.

An incubation temperature of 55°C. was also tried, using beef heart medium under vaseline seal. *Cl. botulinum* A, *Cl. botulinum* B, *Cl. bifermentans*, *Cl. chauvei*, *Cl. edematiens*, *Vibrion septique* and *Cl. sporogenes* developed at this temperature, but not as profusely as at 50°C. *Cl. welchii*, *Cl. tetani*, *Cl. tertium*, *Cl. putrificum* and *Cl. histolyticum* gave no visible growth in five days.

CONCLUSION

The anaerobes investigated with the exception of *Cl. histolyticum* and *Cl. tetani* appear to have a wide temperature range of growth, very little difference being observed between 37° and 50°C. In many cases growth was more rapid at the higher temperature. Some anaerobes will develop at 55°C., but the growth is somewhat retarded.

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THE BACTERIOPHAGE IN RELATION TO SALMONELLA PULLORA[1] INFECTION IN THE DOMESTIC FOWL

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It has been observed by Hankin (1896), Twort (1915), d'Herelle and his associates (1922), and others that Berkefeld filtrates of stools, tissues, and other cultures would kill and dissolve young cultures of dysentery and many related organisms. A transfer of one of these dissolved cultures to a new culture would cause it in turn to dissolve. Thus this lytic or dissolving principle could be transferred from generation to generation. These bacteriolytic filtrates or their subsequent generations are called, in our present literature on the subject, bacteriolytic agents, bacteriolysants, or bacteriophages. The process by which these filtrates dissolve many organisms is known as the d'Herelle phenomenon or bacteriophagy.

Since d'Herelle's (1922) early work on the bacteriophage had so much to do with the identification of the lytic principle in relationship to the organisms of the intestinal groups of bacteria, originating in disease and in healthy individuals of both the human and animal subject, together with the study of specific avian pathogens, it was considered of importance from the standpoint of comparative pathology, to seek for a lytic principle in the domestic fowl against *Salmonella pullora* (*Bacterium pullorum* Rettger). This organism is of great economic importance to the poultry industry and should be studied and eliminated from laying hens, thus protecting the progeny and improving the food supply for human consumption.

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[1] *Bacterium pullorum* Rettger.

The organisms especially studied were those of dysentery (d'Herelle, 1917), typhoid and paratyphoid, (d'Herelle, 1919a) fowl typhoid, (d'Herelle, 1919b, c) and pest and barbone (d'Herelle, 1921). D'Herelle found, when examining the stools of dysentery patients, especially from convalescents, that a suspension of Shiga bacilli became limpid, and later found that this unusual condition was due to a lytic principle, which could be transmitted in series, its lytic activity or virulence, as d'Herelle styled it, being increased from generation to generation. He was of the opinion that the recovery of these dysentery patients was due to the action of this bacteriophage upon the intestinal infection *in vivo.* From this work and from the work of McKinley (1922, 1923), Gratia and de Namur (1922), Piorkowski (1922), Watanabe (1922), Davidson (1922a), Kuttner (1923), Beckerich and Hauduroy (1923), Le Blaye (1923), Pauron (1923), King (1923), Lehndorff (1924), Callow (1922), although data and opinions vary, there are indications that the bacteriophage may be used therapeutically. In the hands of many European investigators, especially Beckerich and Hauduroy (1923) and Bruynoghe and Maisin (1921), the bacteriophage has given great promise as a therapeutic reagent. Bruynoghe and Maisin applied the bacteriophage in staphylococcic infections. After injections of 0.5 to 2 cc. of lysate, improvements were noted in twenty-four to forty-eight hours. Gratia claims that in cases of abscesses and furuncles the process of cure is noticeably accelerated by the administration of the lysate (bacteriophage).

The work of Beckerich and Hauduroy is in accord with that of Bruynoghe and Gratia and in conclusion they state that bacteriophagy applied therapeutically will be extensive and varied. On the other hand Callow (1922) finds that a bacteriophage principle transmissible in series against *Staphylococcus aureus* has been demonstrated in the pus of a series of sixteen staphylococcic infections. In six, it proved lytic for one or more heterologous strains, but not for the homologous strain, in spite of repeated tests. This fact would indicate that since the bacteriophage as isolated from the boils is not active against homologous strains of staphylococci, bacteriophagy in this direction as a therapeutic agent is not very encouraging. Kuttner (1923) found a bacteriophage active against a homologous strain of typhoid, isolated during the convalescent stage of the disease, the patient later developing into a chronic carrier. This author states that it is not easy to correlate with such data the supposed therapeutic value of the bacteriophage.

According to Davidson (1922), d'Herelle's phenomenon as related to human dysentery is apparently non-specific, and a bacteriolysant from a patient's stool may not be as active against the organism causing that patient's disease as against other strains. He claims that it does not necessarily play a part in the immunity or defense-mechanism of the body. These non-specific bacteriolysants had no therapeutic effect when administered to twelve young children suffering from bacillary dysentery (Davidson, 1922b).

Davidson's work (1922a) suggests that the bacteriophage or the lytic principle of d'Herelle's phenomenon is an enzyme. The amount of the bacteriolytic enzyme may be increased in several ways by certain external influences which favor the development of lysogenic organisms at the expense of the non-lysogenic. This enzyme not only dissolves organisms but also favors the multiplication of bacteria which produce the enzyme. In this way the bacteriolytic principle is carried from generation to generation.

D'Herelle (1922) made seventy examinations of the excreta of fowls and tested the bacteriophage for virulence against eight different bacterial strains. The cultures selected for avian bacteriophagic action were *B. coli*, *B. dysenteriae* Shiga, Flexner, Hiss, *B. typhi*, *B. paratyphi* A, *B. paratyphi* B, *B. gallinarium*, and the bacterium of barbone. Bacteriophage activity was demonstrated from all excreta studied, some samples showing marked activity for all cultures treated.

From observations made by d'Herelle (1924), in the course of experiments upon avian typhosis he noted a chicken had contracted the disease, and on examination found that the virulence of the intestinal bacteriophage increased quickly to a maximum and the bird recovered. The epizootic ceased abruptly and experiment showed that in the feces of all chickens was to be found a bacteriophage of maximum activity. D'Herelle claims that his experiments with the bacteriophage, in relation to *B. gallinarium* or fowl typhoid infection, confirm the conclusion that immunity to a bacterium is assured at the time when the body contains a bacteriophage virulent for this bacterium. The immunity is exogenous and is contagious.

From Marshall's work (1925) on the d'Herelle bacteriophage, it is noted that this author concurs in the theory that the agent responsible for transmissible bacteriolysis is a living ultra-microscopic entity.

Although there exists confusion in relation to the therapeutic aspects of the bacteriophage problem, yet from the work of both European and American writers it may be inferred that bacteriophagy may be employed in therapeutics. Since d'Herelle and associates have reported success in relationship to fowl typhoid, is it possible to find a bacteriophage lytic for *Salmonella pullora* (*Bacterium pullorum* Rettger)? Would such a lytic principle be of any therapeutic value in the treatment of this disease in young chicks or mature domestic fowls?

With the above information at hand, the writer has planned to investigate materials and tissues of the domestic fowl to determine the presence of a bacteriophage lytic for *Salmonella pullora* and if present, in sufficient amount and activity, to apply it in the treatment of bacillary white diarrhea of chicks, and *Salmonella pullora* infection in the domestic fowl.

ISOLATION OF THE LYTIC PRINCIPLE

Prior to any attempt at isolation of a lytic principle from the fowl, two available strains of the bacteriophage, received from other laboratories, were used. One strain was a portion of d'Herelle's original Shiga bacteriophage, which did not prove active against the *Salmonella pullora* organism after transmission in series some fifty times, in an attempt to adapt the lytic principle to the specific organism. The same was true of a Coli X bacteriophage obtained indirectly from d'Herelle's laboratory. Likewise a lytic principle isolated from the feces of a cow by the writer, which was specific for the colon bacillus, typhoid and paratyphoid organisms, failed to demonstrate any lytic action upon a suspension of the pullorum bacillus.

While working with the above strains, daily attempts were being made to isolate a "phage" from the feces of the domestic fowl. Several birds were obtained from a Massachusetts flock which had been affected with *Salmonella pullora* infection. Prior to testing the droppings of the specimens for the presence of the lytic principle, blood samples were taken and the serum subjected to an examination for the presence of agglutinins specific for the organism of that disease.

D'Herelle's technic for isolation of the "phage" was carefully adhered to. A portion of droppings weighing approximately 5 grams was thoroughly agitated in a 250 cc. Erlenmeyer flask containing 75 to 100 cc. of bouillon. The culture medium used was adopted after several trials of various formulae and hydrogen ion concentrations. Witte's peptone was finally decided upon in preference to d'Herelle's suggestion of Martin's. It was used in 1 per cent content as usual. The completed formula included as well as the peptone 0.5 per cent NaCl, 0.3 per cent Liebig's extract of beef, and distilled H_2O. Various hydrogen ion concentrations were tried and that most suited to the optimum growth of the pullorum organism (7.4 to 7.6) was selected and used exclusively.

TABLE 1

Agglutination test

BIRD NUMBER	SERUM DILUTION					
	1:50	1:100	1:200	1:400	1:800	1:1600
281	–	–	–	–	–	–
5651	++++	++++	++++	++++	++++	+++
7047	++++	++++	+++	+++	–	–
5568	–	–	–	–	–	–
5735	–	–	–	–	–	–
9242	++++	++++	++++	++++	+++	–

The flask and its agitated contents were incubated for four to eight hours further to decompose the mass. Later on this procedure proved unnecessary since results equally as good were obtained with vigorous agitation alone.

The contents of the flask were filtered through filter paper and infusorial earth in order to remove the coarse material, and then passed through a Berkefeld filter of medium porosity into another Erlenmeyer flask. If a bacteriophage were present it should be contained in this clear, "sterile" filtrate. A minute quantity of this supposed "phage" material was added to a fresh suspension of *Salmonella pullora* organisms washed clear with bouillon from a twelve to eighteen-hour agar slant growth. This was incubated for a few hours to hasten the lytic action, again filtered and a small amount of the filtrate added to a new fresh suspension of the organism. In this way the "phage" was transmitted in series through daily filtrations and inoculations.

Several modifications of this method were developed, but since none of them proved so satisfactory and simple, they will not be described here.

Five strains of the bacteriophage were isolated from the droppings of the fowl, each exerting a marked lytic action against the pullorum organism. They demonstrated at first merely an inhibition in the growth of the suspension of organisms and it was not until the sixth series' generation that any definite lytic action was noted, and then it was of but weak potency. These strains were designated by numbers 5651, 7047, 5568, 5735, and 9242 corresponding to the leg band number of the birds (table 1) from which they were isolated.

Strains 5568, 5735, and 9242 at no time showed a strong lytic action even after daily passages through filters over a period of several months. Often times lytic action seemed to be absent, or if present, it was very erratic. Again at irregular intervals the suspensions would show complete dissolution of the organisms. Hope of developing these strains for therapeutic use was finally abandoned after the one hundred and twentieth generation. These erratic actions of the lytic principles might not only be due to a low virulence, but as well to the development of the "secondary cultures" mentioned by d'Herelle. Some few organisms of the suspensions were undoubtedly more resistant and withstood the action of the bacteriophage. There were instances when strains of *S. pullora* in use appeared to develop marked resistance to bacteriophagy. This difficulty was eliminated by using only young, actively growing organisms.

Strains 5651 and 7047 developed an unusual and marked degree of lytic activity after passage through sixty generations. An explanation of this phenomenon is difficult to find. It might be that the stubborn resistance of the *Salmonella pullora* strains to bacteriophagy had been overcome by the lytic principles. Certainly there had been an apparent enhancement in the virulence of the "phages." It is interesting to note that these two strains, which ultimately became markedly potent, were isolated from birds showing a high titre of their respective sera for agglutinins specific for the *Salmonella pullora* organism.

Bacteriophage strains 5651 and 7047 were characteristic of d'Herelle's lytic principle in every respect. They demonstrated very well indeed the "watery, moth-eaten, or pellucid areas" described in his text. In plate 1 agar slant 4 and bouillon tube 1 are controls. Agar slant 4 was produced by a streak inoculation with a loopful of suspension from bouillon tube 1, containing 10 cc. of suspension with 190,000,000 organisms per cubic centimeter, but no bacteriophage. Bouillon tubes 2, 3, and 4 each contained 10 cc. of a suspension of *Salmonella pullora*, 190,000,000 organisms to the cubic centimeter with 0.01 cc. of bacteriophage added to each tube. Immediately after the addition of the lytic principle to bouillon tube 2, a loopful of the suspension was streaked upon agar slant 1 and all tubes incubated. Following three hours' incubation another loopful from bouillon tube 2 was streaked upon agar slant 2 and incubated, and after five hours' incubation a third loopful from bouillon tube 2 was streaked upon a third agar slant 3 and then incubated. After sixteen hours' incubation agar slant 1 showed 8 to 10 watery areas, agar slant 2 numerous, watery areas, and agar slant 3 numerous coalescent watery areas. This work supports the contention that the lytic principle is capable of proliferation.

Moderately active strains of bacteriophage lytic for *Salmonella pullora* were also isolated from 25 to 50 per cent glycerol-saline solution extracts of liver, intestines, and ovary of the fowl. Like results were obtained from an extract of mucous scrapings of the intestinal mucosa.

The action of the lytic principle in relation to closely related organisms corresponded very much to that of the group agglutinins. Lysis of the specific organism was more pronounced than that for closely allied organisms of the same grouping. *Eberthella sanguinaria* was vigorously acted upon by the "phage" strains, while *Eberthella typhi*, *Salmonella paratyphi*, and *Salmonella schötmulleri* varied in reaction from a two plus dissolution of suspension to a bare inhibition of growth. *Escherichia coli* and the *Eberthella paradysenteriae* and *E. dysenteriae* organisms did not demonstrate bacteriophagy.

ENHANCEMENT OF VIRULENCE OR POWER OF BACTERIOPHAGY

There is little doubt but that the therapeutic value of the lytic principle would primarily depend upon its potency. An attempt to enhance the virulence of the bacteriophagic strains would, therefore, seem justified and would promise measurable success, for such a phenomenon had apparently already occurred. The work as demonstrated in plate 1 indicates that the lytic principle, no matter what its nature might be, was capable of proliferation, and because of a close correlation, enhancement in lytic activity should follow. If the results of such an attempt were positive, it would seem that d'Herelle's ultramicrobial theory as to the nature of the lytic principle would have some basis, but yet the enzyme, autolytic theory, which has been advanced by many workers in this country, would still have considerable foundation, and certainly should not be ignored. It is not the purpose of this paper, however, to consider the explanation of that most complicated and difficult phase of the phenomenon.

The plan of operation employed involved the attempt to obtain complete lysis after sixteen hours' incubation of 10 cc. of bouillon, plus varying quantities of the lytic principle and specific organisms. The preliminary work of the experiment would, of course, be the determination of the quantitative virulence of the "phage," or in other words, its minimum lytic dose. It would also be necessary to determine the maximum number of organisms that would be completely destroyed by this minimum lytic dose. Enhancement of the virulence could not be deduced until these two quantitative factors were known.

"Phage" strains 5651 and 7047 were combined, as were also five of the *Salmonella pullora* strains which had been in general use heretofore. Both polyvalent strains of "phages" and organisms were thus being used. At this point the work presented considerable difficulty and there was doubt in the mind of the writer as to whether or not it was practical to proceed. At that time 0.0001 cc. of the "phage" caused complete lysis of a 10 cc. bouillon suspension of 220,000,000 organisms per cubic centimeter. The daily readings always presented a complete dissolution of the suspensions following sixteen hours' incubation, if not immediately upon removal from the incubator, a least within an hour or so after standing at room temperature.

From these results it is indicated that a stock strain of the avian bacteriophage can be carried indefinitely in the laboratory as long as it is properly cared for by repeated transmission through filters and inoculations into media containing fresh, young organisms.

LYTIC ACTIVITY OF SALMONELLA PULLORA BACTERIOPHAGE

Unless the lytic principle isolated from hens was specific for all *Salmonella pullora* strains, or proved bacteriolytic for homologous strains, it would be of no practical therapeutic value.

This laboratory during the spring chick hatching season is active in making diagnoses upon dead chicks. As each strain of *Salmonella pullora* is isolated and identified, it is given an index number, its history recorded, and the culture kept in the bacteriological museum for a period of one year, at the end of which time it is discarded and replaced by an incoming new strain. Available material was thus on hand for the following experiment.

Each strain of *Salmonella pullora* was subcultured several times until it had so adapted itself that an eight to twelve hour agar slant growth produced abundant, fresh organisms for the suspension, to which the "phage" was to be added. A bacterial

TABLE 2

Lytic activity of avian bacteriophage, isolated from domestic fowls for different strains of Salmonella pullora

SALMONELLA PULLORA STRAIN NUMBER	SOURCE OF CULTURE AS ISOLATED FROM CHICK	DATE	NUMBER OF SALMONELLA PULLORA IN 1 CC. OF SUSPENSION	AMOUNT OF BACTERIOPHAGE SOLUTION	DEGREE OF LYSIS
			millions	*cc.*	
5	Unabsorbed yolk	3/11/24	195	0.01	++++
6	Liver	3/12/24	190	0.01	++++
7	Liver	3/13/24	200	0.015	+++
8	Liver	3/13/24	195	0.01	+++
9	Liver	3/13/24	185	0.01	++++
10	Liver	3/15/24	165	0.01	++++
11	Liver	3/15/24	175	0.01	+++
12	Liver	3/16/24	180	0.01	++++
13	Liver	3/17/24	150	0.01	+++
14	Unabsorbed yolk	3/25/24	155	0.01	++++
15	Liver	3/26/24	190	0.01	++++
16	Liver	3/26/24	190	0.01	+++
17	Unabsorbed yolk	3/28/24	195	0.01	+++
18	Liver	3/28/24	180	0.01	+++
19	Unabsorbed yolk	3/28/24	175	0.01	+++
20	Unabsorbed yolk	3/28/24	185	0.01	++++
21	Liver	3/28/24	160	0.01	++++
22	Unabsorbed yolk	3/29/24	173	0.01	+++
23	Unabsorbed yolk	4/ 1/24	185	0.01	++++
24	Liver	4/ 1/24	190	0.01	++++
25	Unabsorbed yolk	4/ 2/24	185	0.01	++++
26	Liver	4/ 2/24	175	0.01	++++
27	Unabsorbed yolk	4/ 2/24	190	0.01	+++
28	Unabsorbed yolk	4/10/24	180	0.01	++
29	Unabsorbed yolk	4/19/24	180	0.01	+++
30	Unabsorbed yolk	4/23/24	180	0.01	+++
31	Unabsorbed yolk	4/26/24	180	0.01	+++
32	Unabsorbed yolk	5/12/24	185	0.01	+++
33	Liver	5/17/24	190	0.01	++++
34	Liver	5/17/24	190	0.01	++++
35	Heart blood	6/ 1/24	190	0.01	++++
36	Heart blood	6/ 1/24	190	0.01	++++

++++, complete lysis.

count was then made and 0.01 cc. of the bacteriophage added to a 10 cc. bouillon suspension of the organisms. Following an incubation period of sixteen hours at 37.5°C., and one to three hours standing at room temperature, the readings were taken and recorded as summarized below. Each lysed suspension was then passed through a Berkefeld candle and the filtrate used as the bacteriophage for the test on the next strain of the organism.

THE THERAPEUTIC VALUE OF THE AVIAN BACTERIOPHAGE

Since the avian bacteriophage, as isolated from hens numbered 5651 and 7047, was actively lytic for all thirty-one strains of *Salmonella pullora* cultures studied, and since such quantities as 0.0001 cc. caused lysis of a 10 cc. bouillon suspension containing 220,000,000 organisms, material was at hand for applying the bacteriophage as an agent in treatment.

Many factors must be considered when attempting to treat and immunize with the bacteriophage, especially when dealing with a disease such as bacillary white diarrhea. Bacillary white diarrhea infection is not only found in the intestines, but is en-encountered as a severe septicemia as well. The chronic form of this disease as seen in adult hens is usually organic and not to any great degree of an intestinal nature.

Therefore, our first problem was to consider the mode of administration of the lytic principle. Injection of the agent would more likely bring about an organic resolution and immunity, while ingestion could hardly be expected to accomplish such results.

EXPERIMENT I

A lot of forty chicks was obtained from a brood whose progenitors had been tested by means of the agglutination test for *Salmonella pullora* infection and pronounced free of that disease. They were treated as follows:

Pen 1. Ten chicks, controls on brooder management. Received 0.5 cc. of sterile physiological salt solution subcutaneously over the pectoral region.

Pen 2. Ten chicks, controls on pathogenicity of the pullorum culture. Received 0.5 cc. of the culture, subcutaneously, over the pectoral region.

Pen 3. Ten chicks, received simultaneous injection of 0.5 cc. of culture and 0.5 cc. of bacteriophage, subcutaneously, over pectoral region.

Pen 4. Ten chicks, received subcutaneous injection over pectoral region of 0.5 cc. of culture. Eight hours later after period of incubation had been instituted, another subcutaneous injection over pectoral region on opposite side of 0.5 cc. bacteriophage.

Results

All chicks in pen 1, serving as controls on brooder management, lived and thrived throughout the experiment and have since been raised to maturity. All those of pen 2, which controlled the virulence of the *Salmonella pullora* culture, succumbed to the infection, beginning to die at the forty-fourth hour following injection. The chicks of pen 3, which received the simultaneous administration of bacteriophage and culture, began to die at the one hundred and seventeenth hour following the injection, while those of pen 4, first having received the culture and, later, after a short incubation period the bacteriophage, began to die at the one hundred and fifth hour.

The strain of *Salmonella pullora* was recovered from all chicks of pens 2 and 4, and from seven chicks of pen 3. It was impossible to isolate the organism from the remaining three chicks of this pen, but a bacteriophage, presumably the one administered, was isolated from the intestinal tract of one of them. From these results it might be interpreted that these three chicks of pen 3 had reacted well to the bacteriophage, the infection being entirely destroyed *in vivo* and death resulting from another cause, such as intoxication from necrotic tissue at the point of inoculation. In some cases this lesion was extensive and it undoubtedly played a part in hastening death.

The results indicate a slight postponement of death and possibly a less acute form of bacillary white diarrhea following the injection of the bacteriophage.

EXPERIMENT II

It was evident that administration of the culture and bacteriophage by means of the hypodermic needle was very severe. It was found impractical to administer therapeutic treatment in such a manner to numerous baby chicks. A second lot of chicks was accordingly treated in the following manner.

Pen 1. Ten chicks, controls on brooder management, received 5 to 10 drops of bacteriophage *per orem* 4:00 p.m. and same quantity 10:00 a.m. the following day.

Pen 2. Ten chicks, controls on pathogenicity of culture, received 5 to 10 drops of culture *per orem.*

Pen 3. Ten chicks, received simultaneously 3 to 5 drops of culture and 5 to 10 drops of bacteriophage *per orem.*

Pen 4. Ten chicks, received 5 to 10 drops of bacteriophage as a preliminary or "immunizing" dose at 4:00 p.m., and the same amount next morning at 10:00 a.m., simultaneously with 3 to 5 drops of culture.

Drinking water, medicated with bacteriophage, was kept before the birds of pens 3 and 4 at all times.

Results

The results were comparable to those of experiment I in that all chicks of pen 1 lived and those of pen 2 began to die at the one hundred and first hour. The chicks of pen 3 began to die at the one hundred and forty-second hour and those of pen 4 at the one hundred and sixty-third hour.

Salmonella pullora was recovered from all chicks examined, but in no instance was the search for the bacteriophage successful.

A slight postponement in death is again indicated. Each chick received at least 75,000,000 organisms and the bacteriophage was called upon for action against a severe artificial infection. No opportunity was given for experimental use of the lytic principle against a natural infection.

SUMMARY

The purpose of this work has been first to study the intestinal contents, fluids and tissues from the domestic fowl, and to locate and identify in these materials a bacteriophage, lytic for *Salmonella pullora* (*Bacterium pullorum* Rettger).

Secondly, methods have been employed for increasing the lytic activity of the avian bacteriophage *in vitro*, to the end of using it as a therapeutic reagent in avian therapeutics.

From the data presented and the observations made, the following conclusions appear to be justified.

1. Tissues of the domestic fowl do contain bacteriophages causing lysis of various strains of *Salmonella pullora.*

2. Up to the present time, although these avian "phages" are lytic for *Salmonella pullora,* it has not been demonstrated that they are specific only for this organism.

3. When agar slant cultures of *Salmonella pullora* are treated with liquid cultures of these avian bacteriophages which are lytic for *Salmonella pullora,* watery, moth-eaten or pellucid areas are demonstrated. This fact, together with the demonstration of the transmission of bacteriophagy for *Salmonella pullora* in series would indicate that the transmissible bacteriolysis is a living ultra-microscopic entity. These reactions of the avian bacteriophage are characteristic of d'Herelle's "Bacteriophagum Intestinale."

4. Although the bacteriophages isolated and studied have demonstrated marked bacteriolysis *in vitro,* the evidence from animal experiments does not indicate that as now prepared and used that they have much therapeutic effect in freeing the bird's body of infection.

5. Bacteriophages actively bacteriolytic for *Salmonella pullora* have been isolated from domestic fowls showing a high agglutinative titre for this organism.

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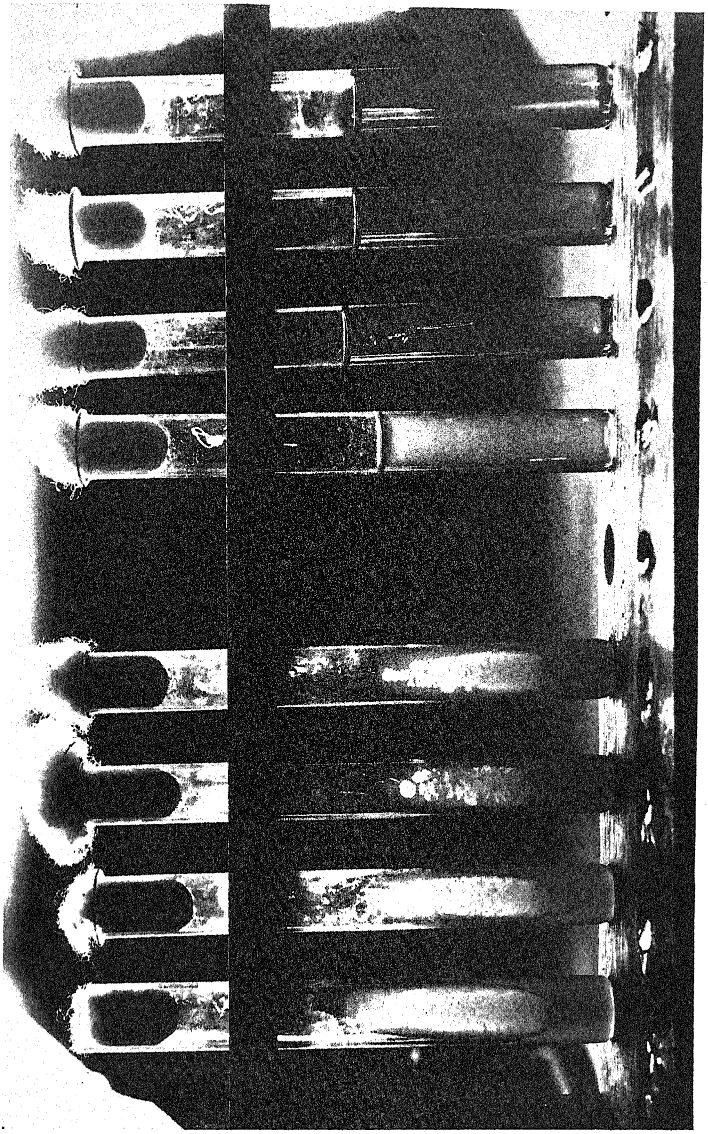
PLATE 1

Agar Slant and Bouillon Cultures of Salmonella Pullora Showing “Watery, Moth-Eaten Areas” and Complete Lysis Indicative of the Avian Bacteriophagic Activity

Agar slant 4 and bouillon culture 1 (reading from left to right) are controls, the former being produced by a streak inoculation with a loopful of suspension from the latter. Bouillon cultures 2, 3, and 4 were each inoculated with 0.01 cc. of the avian bacteriophage and incubated. A loopful of suspension from bouillon tube 2 was streaked upon agar slant 1 immediately after addition of the bacteriophage. Agar slant 2 was produced from bouillon tube 2 in the same manner after three hours' incubation of bouillon tube 2, and agar slant 3 after five hours' incubation of bouillon tube 2. The agar slants and bouillon cultures after sixteen hours' incubation showed the following:

Agar slants	*Bouillon tubes*
Tube 1. 8 to 10 “pellucid” areas	Tube 1. Control suspension
Tube 2. Numerous, “pellucid” areas	Tube 2. Complete dissolution of organisms
Tube 3. Coalescent, “pellucid” areas	Tubes 3 and 4. Supplementary to tube 2.
Tube 4. Control slant	



(Pyle: Salmonella Pullora Infection in Fowl)

THE INFLUENCE OF SODIUM CHLORIDE ON THE COLORIMETRIC DETERMINATION OF pH[1]

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In the course of the investigation of various halophilic bacteria it became of interest to determine the magnitude of the sodium chloride error in the colorimetric estimation of pH. Various investigators (Clark, 1925) have estimated salt errors at low concentrations (1 M or less) while both Wells (1920) and Ramage and Miller (1925) have studied the salt errors of cresol red over fairly wide ranges of salt concentration.

The object of this work was to ascertain the errors in measuring colorimetrically the pH of solutions 1, 2 and 3 M with respect to sodium chloride using the sulfon phthalein series of indicators. The general procedure consisted in buffering the salt solutions, adding indicator and adjusting to the same color as that given by an identical buffer containing no salt. The pH of each solution was then measured electrometrically and the difference between the two readings taken as the salt error.

The standard buffer solutions proposed by Clark and Lubs (Clark, 1925) were prepared in M/5 concentration from specially purified materials and preserved in glass stoppered Pyrex glass containers. The sodium hydroxide solution was prepared carbonate free from sodium amalgam and preserved in an appropriate manner. The buffer solutions were made up to the recommended M/20 concentration immediately before use.

The following is given as an example of the method finally adopted. First the standard buffer solution was made up to a suitable pH, a 10 cc. portion measured into a comparator tube and indicator added. An identical buffer solution 1, 2 or 3 M with respect to salt, as desired, was made up and 10 cc. measured into another comparator tube and the same quantity of indicator added. With the sulfon phthalein series of indicators the salt-buffer solutions invariably gave colors further toward the acid side than did the non-salt buffers.

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[1] Presented at the twenty-seventh annual meeting of the Society of American Bacteriologists, Madison, Wisconsin, December 29 to 31, 1925.

Three solutions of M/5 sodium hydroxide 1, 2 and 3 M with respect to sodium chloride had previously been made up. To the 10 cc. of salt buffer solution was added from a micro burette sufficient of the appropriate salt-sodium hydroxide solution to give a color match between the salt and non-salt buffer. No more than 0.3 cc. of sodium hydroxide was ever required which would amount to no more than 3 per cent error in the buffer and indicator concentrations. This is well within the limits of the error in the colorimetric procedure. As soon as a color match was obtained between the two the pH of each was measured electrometrically. At least three determinations were made on each of the three salt concentrations with each indicator. To make the work as comprehensive as possible measurements were made over the useful pH range of each indicator. These various determinations of salt error usually agreed within 0.05 pH for each salt concentration. The average of the various results for these indicators is presented in table 1.

TABLE 1

Salt errors of the sulfonphthalein indicators

INDICATOR	CORRECTION		
	1 molar	2 molar	3 molar
Thymol blue (alkaline range)	−0.22	−0.29	−0.34
Cresol red	−0.28	−0.32	−0.37
Phenol red	−0.21	−0.26	−0.29
Brom thymol blue	−0.19	−0.27	−0.29
Brom cresol purple	−0.26	−0.33	−0.31
Brom cresol green	−0.26	−0.31	−0.29
Brom phenol blue	−0.28	−0.37	−0.43
Thymol blue (acid range)	−0.10	−0.13	−0.12
Methyl red	−0.04	−0.01	+0.12

It is of interest to note that the salt errors of the sulfonphthalein series of indicators are of the same sign and approximately the same magnitude. Methyl red which has the smallest error while not belonging to this series, was included for the sake of completeness.

The results are in accord with the observations of Wells that the greatest effect is obtained with the lower concentrations of salt, while above 1 M the further salt additions give relatively slight increases in salt error. The data for cresol red are in good agreement with the extrapolated observations of Ramage and Miller. These data are useful in colorimetric estimations in solutions of 1 to 3 M (6 to 18 per cent) salt since for most bacteriological purposes, using the tube or spot plate method, the subtraction of 0.3 pH unit from the observed values will bring the colorimetric into conformity with the electrometric pH within the probable error of the former determination.

During the progress of this work attempts were made to secure more accurate color matches by making comparisons of the salt and non-salt indicator colors in a colorimeter. It was never possible to obtain a perfect match in the divided field although no difficulty was experienced using tubes and a comparator block. This observation suggests that a rather marked difference exists in the light transmissive properties of the salt and non-salt indicator solutions. It would seem that a spectro-photometric investigation might yield interesting results regarding the nature of the salt effect.

SUMMARY

1. The salt error of the sulfonphthalein series of indicators has been measured at 1, 2 and 3 M salt concentrations.
2. By subtracting 0.3 unit from the observed colorimetric pH results may be obtained for solutions containing 6 to 18 per cent salt which are in conformity with the electrometric data.

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CONDUCTIVITY AS APPLIED TO STUDIES OF BACTERIAL METABOLISM

II. PARALLELISM BETWEEN AMMONIA AND CONDUCTIVITY IN NUTRIENT GELATIN CULTURES OF PUTREFACTIVE ANAEROBES[1]

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In a previous paper the possible value of conductivity measurements in studies of bacterial proteolysis was discussed and data were presented demonstrating a direct proportionality between conductivity change and ammonia production in the case of two anaerobes (*C. flabelliferum* and *C. sporogenes*). The investigation has been extended to a representative group of strongly proteolytic anaerobes to ascertain whether a correspondingly definite relationship exists for these organisms also.

Nutrient gelatin was selected as the medium for this study because of its universal use and because it lends itself readily to conductivity measurements. Bacto nutrient gelatin (dehydrated), adjusted to pH 8.0, was employed.

Following are the cultures and their sources:

C. sporogenes	Army Medical School
C. flabelliferum	Cudahy Laboratory
C. bifermentans	Lister Institute
C. histolyticum	Lister Institute
C. Reading	Lister Institute
C. parasporogenes	Lister Institute
C. putrificum	Yale Bacteriological Laboratory

Invigorated cultures were inoculated into 25 cc. portions of medium in large culture tubes and incubated in hydrogen at 20° and 37°C.

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[1] Presented at twenty-seventh annual meeting of the Society of American Bacteriologists, Madison, Wisconsin, December 29 to 31, 1925.

Measurements of electrical conductivity were made at 30°C. Ammonia was determined by the Folin method using 1 cc. samples. Control tubes of sterile media were analyzed and appropriate conductivity and ammonia blanks subtracted from all subsequent determinations. Changes in specific conductivity in reciprocal ohms (Δk), and changes in ammonia nitrogen were tabulated. To facilitate interpretation of the data Δk was plotted against milligrams of ammonia nitrogen per 100 cc. (ΔN) for each organism at each temperature. These $\Delta k - \Delta N$ plots in all cases appeared to be straight lines.

The curves for all organisms except *C. histolyticum* had the same slope. This line gave the equation

$$\Delta N = \Delta k \times 1.95 \times 10^4$$

This is the same proportionality constant which the writers previously found for *C. flabelliferum* and *C. sporogenes*. *C. histolyticum* also gave a linear relationship which was better expressed by the slightly different constant 1.87×10^4.

To gain an idea of the applicability of these constants, data have been tabulated showing Δk and ΔN (both calculated and observed) and the per cent deviation of the calculated from the observed value for each culture. The data for *C. sporogenes*, *C. flabelliferum* and *C. bifermentans* are given for 20° only since the first two have been reported previously for 37°C. and the last was not run at 37° owing to an oversight.

The calculated ΔN values in tables 1 to 6 were computed using the constant 1.95×10^4 while those in table 7 were obtained with the constant 1.87×10^4. Had the former constant been used for *C. histolyticum* the deviations would have been within 10 per cent but in all instances the calculated values would have been greater than the observed. It will be noted that in most cases where the ammonia value was very low large percentage deviations were obtained. This is not indicative of any inherent weakness in the conductivity method but is explicable by the fact that analyses for ammonia were invariably carried out on 1 cc. samples in order to maintain identical conditions for all determinations. Obviously slight unavoidable titration errors and possible variations in the initial composition of the media will yield relatively large errors when the ammonia content of the sample is low. If one were interested in obtaining measurements of such low concentrations of ammonia it would doubtless be possible by the choice of larger samples and by careful observation of the initial conductance to reduce the deviations to a minimum.

TABLE 1

Ammonia conductivity relationships.—C. sporogenes, 20°C.

AGE	Δk	ΔN		PER CENT DEVIATION
		Calculated	Observed	
days				
2	0.9×10^{-3}	17.5	20	−12.5
4	4.6×10^{-3}	90	86	+4.6
6	8.3×10^{-3}	162	158	+2.5
10	18.3×10^{-3}	357	363	−1.6
16	21.1×10^{-3}	412	433	−2.1

TABLE 2

Ammonia conductivity relationships—C. flabelliferum, 20°C.

AGE	Δk	ΔN		PER CENT DEVIATION
		Calculated	Observed	
days				
2	1.1×10^{-3}	21	14	+50.0
4	8.1×10^{-3}	158	162	−2.5
6	12.3×10^{-3}	240	248	−3.2
10	17.6×10^{-3}	343	363	−5.5
16	22.5×10^{-3}	438	458	−4.4

TABLE 3

Ammonia conductivity relationships—C. bifementans, 20°C.

AGE	Δk	ΔN		PER CENT DEVIATION
		Calculated	Observed	
days				
2	2.7×10^{-3}	52	46	+13.0
4	8.5×10^{-3}	166	154	+7.8
6	16.3×10^{-3}	318	319	−0.3
10	22.6×10^{-3}	441	438	+0.7
16	26.8×10^{-3}	523	525	−0.4

TABLE 4

Ammonia conductivity relationships—C. Reading

AGE	20°C.				37°C.			
	Δk	ΔN		Per cent deviation	Δk	ΔN		Per cent deviation
		Calculated	Observed			Calculated	Observed	
days								
2	0.8×10^{-3}	15	13	+15.4	13.0×10^{-3}	254	255	−0.4
4	4.0×10^{-3}	78	79	−1.3	17.0×10^{-3}	332	331	+0.3
6	8.4×10^{-3}	164	159	+3.1	25.0×10^{-3}	488	483	+1.0
10	15.2×10^{-3}	296	309	−4.2	29.3×10^{-3}	572	565	+1.3
16	22.6×10^{-3}	441	461	−4.3	30.2×10^{-3}	588	599	−1.8

TABLE 5

Ammonia conductivity relationships—C. parasporogenes

AGE	20°C.				37°C.			
	Δk	ΔN		Per cent deviation	Δk	ΔN		Per cent deviation
		Calculated	Observed			Calculated	Obsevred	
days								
2	0.8×10^{-3}	15	12	+25.0	13.6×10^{-3}	266	263	+1.1
4	5.2×10^{-3}	102	122	−16.4	21.2×10^{-3}	414	402	+3.0
6	11.5×10^{-3}	224	218	+2.8	23.2×10^{-3}	452	471	−4.0
10	21.1×10^{-3}	412	409	+0.7	26.7×10^{-3}	521	517	+0.8
16	25.2×10^{-3}	492	489	+0.6				

TABLE 6

Ammonia conductivity relationships—C. putrificum, 37°C.

AGE	Δk	ΔN		PER CENT DEVIATION
		Calculated	Observed	
days				
6	5.3×10^{-3}	103	94	+9.6
10	17.5×10^{-3}	341	335	+1.8
16	19.6×10^{-3}	382	390	−2.0

Note: Cultures of *C. putrificum* did not show digestion at 37° until 6 days and at 20°C. no appreciable digestion was noted up to 16 days.

While it has been shown that change in conductivity exactly parallels change in ammonia content it must be borne in mind that the measured conductivity is the sum of the conductances of the ammonium ions and all other ions which are formed, both basic and acidic. It is not probable that any considerable quantity of basic ion other than ammonium would result from the proteolytic decomposition of gelatin. The fact that the same constant serves equally well for computing ammonia from conductivity for the first six anaerobes indicates that the average conductivity of the various acidic ions produced is practically identical although this does not necessarily mean that the acids are the same either in character or quantity. It would appear, therefore, that the acidic ions in cultures of *C. histolyticum* have a greater average conductance than the acidic ions of the other anaerobes. This probably implies that *C. histolyticum* produces acids of a lower mean molecular weight than do these other anaerobes.

TABLE 7

Ammonia conductivity relationships—C. histolyticum

AGE	20°C.				37°C.			
	Δk	ΔN		Per cent deviation	Δk	ΔN		Per cent deviation
		Calculated	Observed			Calculated	Observed	
days								
2	6.4×10^{-3}	119	118	+0.8	27.6×10^{-3}	516	494	+4.5
4	19.5×10^{-3}	365	354	+3.1	24.3×10^{-3}	455	423	+7.0
6	24.1×10^{-3}	450	442	+1.8	32.3×10^{-3}	605	605	0.0
10	28.3×10^{-3}	530	536	−1.1	39.4×10^{-3}	737	723	+1.9
16	39.9×10^{-3}	747	758	−1.4	38.1×10^{-3}	713	712	+0.1
2	6.0×10^{-3}	112	118	−5.1	28.5×10^{-3}	532	525	+1.3
4	18.4×10^{-3}	344	333	+3.3	33.7×10^{-3}	630	617	+2.1
6	23.7×10^{-3}	442	407	+8.6	36.4×10^{-3}	680	675	+0.7
10	28.3×10^{-3}	530	539	−1.6	47.7×10^{-3}	894	928	−3.6
16	36.5×10^{-3}	682	701	−2.7	42.1×10^{-3}	786	793	−0.9

The relationship already shown to exist between conductance and ammonia for two anaerobes seems to be generally true for organisms of this type. Many possible applications to metabolic studies will suggest themselves.

SUMMARY

1. The conductivity method as a measure of ammonia, formerly proposed and employed in studies of *C. flabelliferum* and *C. sporogenes* by the writers, has been applied to cultures of six other representative proteolytic anaerobes.

2. The relationship expressed by the equation,—change in NH_3N = change in conductivity $\times 1.95 \times 10^4$,—held true to within 10 per cent for cultures grown at 20° and 37°C.

3. *C. histolyticum* gave values better expressed by the constant 1.87×10^4.

4. The method promises to be of considerable use in metabolic studies.

A METHOD OF STAINING BACTERIAL FLAGELLA

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It has been thought useful to give the details of a modification of Muir's method of demonstrating the flagella of bacteria, recently developed in this laboratory. The method has proved successful in staining the flagella of about one hundred strains of soil bacteria and has been tested on other common types such as *B. coli*, *B. fluorescens*, and *B. mycoides*. The process has given much more certain results than any of the standard methods tested and possesses the advantage that very little precipitate remains on the film surrounding the bacteria.

CULTURES

The best results are obtained with twenty-four-hour to three-day old slope cultures on nutrient agar or some other medium suited to active growth. (Nutrient agar in the case of the soil organisms upon which the stain was successful is composed of pepton 5 gr., Lemco 3 gr., and agar 15 gr. per litre of water; pH adjusted to 7.3.) Suspensions are made in sterile distilled water in a watch glass and left for twenty minutes to half an hour at room temperature, during which time the organisms if active will be washed free of slime. They should be examined for motility in the fresh state, immediately before the film is made.

SLIDES

Clean slides are kept in ammonia-alcohol; for use they are dried with a clean duster and flamed by passing 24 times through the Bunsen at the level of the top of the inner cone. They are then put into an oven at 45° to 50°C. to cool, flamed side uppermost.

THE FILM

One large loopful of the suspension is placed near one end of the slide. A strip of unsized paper (e.g., typing paper) of a width less than that of the slide is lowered on to the loopful and drawn gently down the slide and off towards the operator. The slide is then put into the oven to dry. The paper strip is convenient in that it allows of a very thin film which dries off quickly.

THE MORDANT

This is made up of the following solutions which when stored separately have kept without deterioration for a year in this laboratory.

Solution 1

Potash Alum—Saturated aqueous sol.	5 cc.
Tannic acid—20 per cent aqueous solution. (A few drops of $CHCl_3$ must be added to this if a large quantity is made up)	2 cc.
Mercuric chloride—Saturated aqueous solution	2 cc.

Solution 2

Basic fuchsin—Saturated alcoholic solution

For use 0.4 cc. of the fuchsin solution is added to solution 1; the solutions are mixed by rapid rotation of the test tube, when a precipitate will form. The solutions must be freshly mixed for each batch of slides as the mixture deteriorates after twenty-four hours.

About 0.5 cc. is allowed to act on each slide for ten minutes at room temperature.

STAIN

Wash off the mordant with a gentle stream of distilled water. When no more fine precipitate is removable apply a few drops of Ziehl's carbol fuchsin and leave for five or ten minutes. Wash off with tap water.

THE RÔLE OF STARCH IN THE CRACKING OF CHOCOLATE CREAMS[1]

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The cracking or "blowing" of the coating of chocolate creams during storage causes much loss in the confectionery trade. The damage is not limited to the loss of material and labor but often includes injury to a good trade name since the spoilage is not always apparent until the goods have been distributed to the retail trade or final consumer; therefore, any contribution to the explanation of the cause or spread of this trouble should be of interest and value.

As judged by our experience this spoilage occurs in all grades and varieties of chocolate creams, but is much less frequent in the hand moulded pieces than in the machine moulded. However, it is sometimes found in hand moulded creams of the highest grade. The thickness of the coating is an important factor in this type of spoilage since there is little if any change in the centre itself and it is only when the coating cracks and allows the liquid centre to leak out that it is noticeable. It, therefore, follows that regardless of all other factors a thinly coated piece is more likely to spoil than a heavily coated piece and it is due in part to their heavier coating that the hand moulded pieces spoil less often.

The spoiled pieces show two types of spoilage, that in which only small droplets of syrup appear on the surface which when wiped off show pin-holes in the chocolate coating, and that type in which there is a distinct crack near the base, often encircling the entire piece and allowing the syrupy centre to ooze out and smear the surrounding pieces. The first variety of spoiled creams show no fermenting organisms when the area around the leak is examined by cutting out a core including the hole and placing in glucose broth and incubating anaerobically. Apparently, the pinholes are mechanical defects in the coating. The second variety, the cracked creams, show the presence of bacteria, capable of fermenting glucose under anaerobic conditions, in the majority of the centers tested.

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[1] Presented before the twenty-seventh annual meeting of the Society of American Bacteriologists, Madison, Wis., December 29 to 31, 1925.

Since our aim has been to eliminate the trouble rather than to identify these organisms, we have made comparatively little study along that line. Partial study was made, however, of thirteen cultures isolated from seven different lots of candies, coming from three different sources, one glucose and one albumen preparation. They were isolated from fermentation tubes which had shown active gas formation under anaerobic conditions. Of these thirteen, ten had the same general characteristics except that three failed to ferment lactose. They may be described as follows: Round ended bacillus, occurring singly or in chains of two or three, size 4 by 1μ; spore forming, spores oval, 1 by 2μ; ferments glucose under both anaerobic and aerobic conditions, ferments lactose; coagulates milk and peptonizes the coagulum; liquefies gelatin, stratiform; grows in broth forming pellicle; gelatin colonies raised, entire, circular, later sunken, grummose. Although these characteristics are not sufficient to afford exact identification they place the organisms definitely in the *B. subtilis* group.

The original source of infection of the candy may be practically any one of the ingredients although at the present day the necessity of high grade materials is so well recognized that they are ordinarily obtainable. Routine examinations of confectioners' glucose and of the albumen preparations offered the trade, seldom show the presence of glucose fermenting organisms. However, such materials are at times infected with organisms capable of fermenting glucose and their use is responsible for the spoilage of the candies. Weinzirl (1922) has reported a case in which it was possible to trace the blowing of chocolate to egg albumen and he identified the causative organism as *Bacillus sporogenes.* More recently, Hill (1925) has reported chocolates spoiled by an organism which she identifies as *Clostridium multifermentans,* introduced by an albumen preparation. Similarly, there has come to our notice a case of blowing in hand moulded chocolates, which was traced to the egg albumen which had been unduly slow in dissolving. Sugar itself has recently been found by the Bureau of Chemistry to harbor yeasts which at times are responsible for the trouble and other materials such as fruits are sometimes the source of the infection.

Our interest in the "blowing" of chocolate creams was aroused about five years ago when three manufacturers came to us with the same story of unusual losses from cracked or blown goods. Since then numerous instances have given us excellent opportunities for further investigation of this problem.

Bacteriological examinations were made of the raw ingredients which entered into the composition of the creams to see if organisms, capable of producing fermentation under anaerobic conditions could be found, but they proved to be either sterile or to contain extremely few organisms.

On the other hand examination of the starches used in the moulds showed them to contain organisms similar to those found in the spoiled pieces, and their number ranged from 3 to 33 per gram. The fresh or new lots of starch showed the smaller numbers while those starches which had been used showed the higher numbers. The number of aerobic bacteria often ran into the thousands with the used starches; moulds were always abundant, and occasionally yeasts were present.

Proof that the use of badly infected starches greatly increases the numbers of glucose fermenting organisms was readily established by testing batches of fondant before moulding and the moulded centres. An example may be cited in which the fondant showed the absence of such bacteria while the centres moulded from the fondant showed in the six examined, 0, 4, 8, 8, 12 and 12 bacteria per piece. The starch in use on the mould gave the following differential bacterial counts, per gram, aerobes 12,000, and anaerobes or facultative anaerobes, 125, of which 33 fermented glucose. Further and more practical proof is given in that the trouble disappeared when the infected starch was discarded.

Studies of several other outbreaks of "blown" chocolate have impressed us with the fact that since the blowing of chocolates is often not detected until the materials used in their manufacture have been consumed, an examination of the moulding starch should always be made. If the infecting material is still in use it can be discarded, but the trouble will not cease if the starch has become badly infected in the meantime. If, on the other hand, the infecting material has already been consumed in the process of manufacture, examination of the material in use will throw no light on the problem and examination of the starch is necessary to obtain an idea as to whether or not a continuance of the spoilage may be expected. A slight infection of the starch may be eliminated sometimes by a partial replacement with new material which acts as a diluent of the infecting organisms but when the infection is heavy or of long standing the starch should be completely replaced.

To summarize: We find that "blown" chocolates usually contain a bacillus which is capable of fermenting glucose under anaerobic conditions; that while this organism probably enters through the use of some infected ingredient this cannot always be established when the spoilage is apparent; that, during the period of the use of the infecting material the moulding starch becomes infected and acts as a secondary infecting agent long after the primary agent has been eliminated by use; and that, the infected starch must be discarded to secure final and complete elimination of the infection.

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STUDIES IN MICROBIC HEREDITY

VIII. THE INFECTIVITY AND VIRULENCE OF A FILTRABLE PHASE IN THE LIFE HISTORY OF B. FUSIFORMIS AND RELATED ORGANISMS

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INTRODUCTION

A little more than seven years ago we reported the presence of a filtrable organism in the blood stream of a patient having a pyemia, due to *B. fusiformis* and related microörganisms (Mellon, 1919a). Although at that time very considerable additional work was done on the filtrability of the fusospirillary complex of organisms present in this patient it was not reported,—partly for the reason that the filtrates were cultivated with great difficulty, and partly because at that time it was not generally believed that common bacteria possessed filtrable phases.

Since then the bacteriophage has come on the scene in a way that leaves no doubt that at least certain of the enteric group have filtrable phases. Reports of filtrability of the tubercle bacillus and other organisms represent a confirmation of isolated cases, instanced not only by my own work, but by studies like that of Fontes and Almquist. Fontes (1909) reported that the tubercle bacillus was filtrable, and Almquist (1916) filtered *B. typhosus* and cultivated a minute variant typhoid bacillus which he called *B. antityphosum;* and now we can surmise that the early filtration experiments which so greatly delayed the discovery of the real cause of scarlet fever represented nothing more than a demonstration that this streptococcus had a filtrable phase in its life history.

But Miss Evans (1925) of the Hygienic Laboratory has placed the filtrability of streptococci beyond the realm of surmise by showing that a green streptococcus and the so-called filtrable virus of encephalitis lethargica are two reversible phases of the same organism, which in addition has a third or diphtheroid phase. All of these may grow independently under proper conditions. Although the first two of these agents have been previously identified with the infection by others, each has been considered by its sponsors as the primary "causus morbi" and as a result two sharply contrasted points of view obtain.

The promise of Miss Evans' work is the reconciliation of the opposed points of view, which has been impossible heretofore on the basis of the old monomorphic conceptions of bacteria. In light of these studies our own work, done nearly ten years ago, may not seem so novel as it would had it been reported at that time. However, infection in experimental animals with the fusiform bacillus and related organisms has rarely, if ever, been accomplished. The discovery, then of an infective and filtrable phase for them definitely correlates virulence with a polymorphic life cycle, which does much to clarify the pathogenesis of diseases thought to be caused by these organisms.

SALIENT POINTS OF OUR FORMER STUDY OF B. FUSIFORMIS. THE BASIS OF THE INOCULATION EXPERIMENTS

It will be recalled that our original study of case J. B. showed that from a renal abscess there was isolated a true anaerobic Gram-negative *B. fusiformis* which was associated with a spirillary non-motile threadlike branching organism. On isolation the latter proved to be a streptothrix or, more precisely in this instance, a vibriothrix. No motile spirochetes were observed in the pus of this case. From the pus obtained by lung puncture the vibriothrix was apparently present in pure culture, and it was not until months later that multiple pleural sinuses developed, in the pus of which a typical Vincent's flora appeared. A filtrable phase of a coccus was present in the blood, which when grown out on a slant as an "adult" coccus no longer passed through a filter. Its presence in the blood in filtrable form was repeatedly demonstrated.

In addition there was described a granular aerobic diphtheroid organism which morphologically was transitional between the fusiform and the vibriothrix. It is this organism which yielded the filtrable gonidia which we pictured, and to which we alluded especially on page 518 in the statement that "Subcutaneous injection of the filtrates into animals has shown results under certain conditions." The pus from which the organisms were isolated and the organisms themselves, when injected subcutaneously into rabbits and guinea pigs, reacted negatively. This is the usual outcome with organisms of this group.

In figure 1 is shown this organism in its gonidial stage, the very numerous granules occurring chiefly in cultures six to eight hours old on fresh blood agar. (Lateral and terminal germination of gonidia at *a*, figure 1.) They were not observed when the organism was grown on other media. These granules passed a Berkefeld N candle and resisted all attempts at artificial cultivation. That is to say they never grew out as the typical bacillus that gave rise to them, although as I described, an abortive coccoid type of germination appeared to occur. Both anaerobic and aerobic technique was employed on both fluid and solid media and at different temperatures.

Our attempts at cultivation and the incomplete results obtained led to our inoculation experiments with the hope that in the body of the animal the conditions for the complete germination of these filtrable forms might be found.[1] Injected subcutaneously into guinea pigs and rabbits the filtrates were quite negative, but intravenous injection into young rabbits resulted in their gradual loss of weight, a moderate temperature and subsequent death; but inasmuch as the cultures from the animals were negative the results could not be considered final.

We then conceived the idea of growing the gonidial filtrates in symbiosis with a saprophytic organism previous to their inoculation. The latter will be known as organism X.

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[1] It will be recalled that the central theme of these studies has grown up around the working hypothesis that bacteria elaborate special growth forms of various sorts, most of which have been collectively expressed as "involution forms" because they were supposed to be incapable of germination. The selection of suitable environments for their germination has been accomplished so often as to virtually abrogate the original meaning of this term.

EXPERIMENTAL

A. *Filtrates of the diphtheroid bacillus*

Experiment 1. Gonidial filtrates of the diphtheroid bacillus were grown at 37° in symbiosis with organism X, and 0.5 cc. inoculated subcutaneously into a 500-gram guinea pig. The inoculation was made immediately beneath the skin leaving an elevation of the shaved area. Six guinea pigs were so inoculated at various intervals with filtrates made from eight and twelve hour cultures showing numerous gonidia (fig. 1). The following data are characteristic for all of them.

Results. After twenty-four hours a small amount of local edema which in forty-eight hours gave way to necrosis without sloughing at this stage. By seventy-two hours marked infiltration of the surrounding tissues and frequently an early purulent exudate were noted underneath the necrosed skin.

Microscopically short fusiform bacilli, coccoid forms and perfectly typical fusiform bacilli with spiral threads were observed (figs. 2 and 3). The morphologic picture recalled that of a Vincent's angina lesion, but actual spirochetes were not demonstrated. Anaerobic Gram-negative *B. fusiformis* was isolated from the lesions, which became rapidly progressive. Usually after forty-eight hours organism X could no longer be isolated. The necrotic skin sloughed, leaving an area of thickened necrotic overhanging margins. The latter were bathed in purulent exudate, particularly beneath them and spreading into the surrounding tissues.

B. *Control series*

Experiment 2. All together fourteen pigs were injected with broth cultures of organism X grown twelve to fifteen hours at 37°C. Of these five received organism X in purity. The others were injected along with filtrates suspected of containing gonidia.

Results. With one exception the results were quite negative in all fourteen animals. By this I mean that although in from twenty-four to forty-eight hours there was some local edema and perhaps necrosis present—as occurred also in the test animals at this stage—the lesions did not progress, smear and culture examinations were negative except for the injected organism, and practically all traces of the inoculation soon disappeared. In the one animal that proved the exception the small amount of pus containing granular, fusiform-like bacilli soon disappeared, and the lesion quickly healed. It seemed probable that this result was to be explained by the previous presence of skin diphtheroids that were thrown into a gonidial phase by the local inflammation resulting from organism X. But the thirteen other negative controls showed clearly that organism X alone is incapable of developing active fusiform lesions. Its morphology rendered it easily distinguishable from *B. fusiformis*.

Experiment 3. The diphtheroid culture grown on plain agar where it did not produce gonidia, at least in appreciable numbers, was emulsified in broth and grown symbiotically with organism X for from twelve to eighteen hours at 37°. Injected 1 cc. subcutaneously into three guinea pigs.

Results. Quite negative after two weeks daily observation, showing clearly that the non-filtrable stage of the organism, even in appreciable numbers and when associated with organism X, was avirulent.

Experiment 4. One cubic centimeter of gonidial filtrate *alone* was given on several occasions subcutaneously to two guinea pigs.

Results. Always negative.

Experiment 5. *a.* Gonidial filtrate was heated ten minutes at 55°C. It was grown as before with organism X and 0.5 cc. injected subcutaneously into a guinea pig.

b. The sterile filtrate of organism X plus the gonidial filtrate was injected subcutaneously into a guinea pig.

Results. Negative in both *a* and *b*.

Experiment 6. The other negative controls comprised three pigs that were inoculated with organism X grown with other stages of the polymorphic complex, e.g., with the vibriothrix.

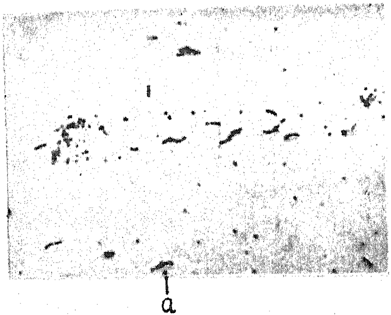

fig. 1

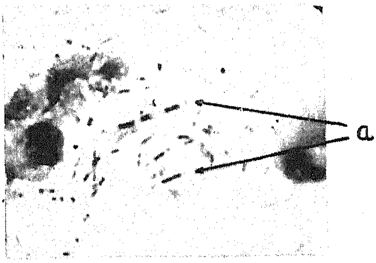

fig. 3

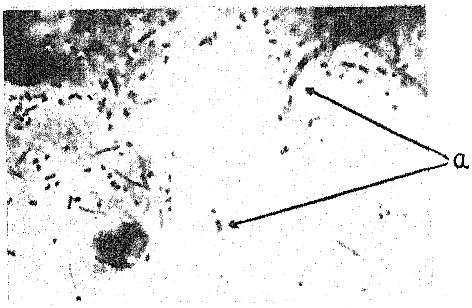

fig. 2

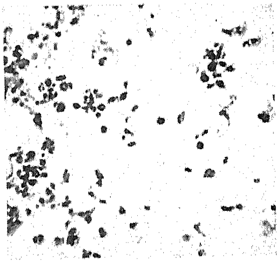

fig. 4

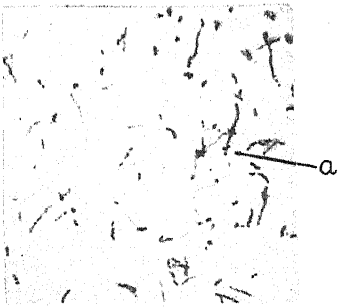

fig. 5

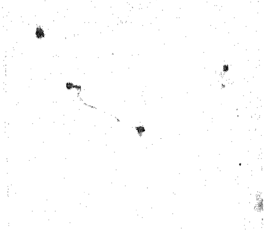

fig. 6

C. Inoculations with the filtrates of the vibriothrix

Experiment 7. Organism X and the vibriothrix were grown together on an agar slant which resulted in a marked granular transformation of the threads. These were emulsified in broth, filtered through a Berkefeld N and the filtrate incubated as usual with organism X.

Results. On February 12, 1918, 1 cc. of the culture was injected subcutaneously into a pig. In forty-eight hours a few granular bacilli and fusiforms could be detected, but on February 18, 1918 pus again appeared at the border of the lesion which contained very numerous fusiform bacilli and numerous other forms indistinguishable from *B. thetoïdes* (figs. 2 and 3 at *a*). After a copious discharge of mucopurulent exudate the lesion went on to complete healing by February 23, 1918. Hair grew over it and the pig appeared quite normal but its original weight of 465 grams remained stationary.

After an interval of six weeks, on April 2, 1918, the original lesion again appeared, but in place of the fusiforms which originally had dominated the morphologic picture giant coccus forms resembling the cultural "coccoids" of the bacillus appeared (fig. 4). Many of these seemed to be the origin of filamentous forms. Many of the latter were present. They often branched dichotomously and yielded lateral and terminal gonidia as seen in the pure culture of figure 5.

On April 7, 1918, the lesion was again nearly healed only to break down on April 15, 1918, being then accompanied by rapid extension into neighboring tissues. At this time 5 cc. of heart blood was withdrawn for culture, which appeared to result in the death of the animal three hours later.

Autopsy protocol. Hemorrhagic exudate was noted at the site of inoculation (left side). Extending from this point to the ribs above and the knees below the subcutaneous tissues were markedly thickened and edematous and quite adherent to the skin. The hyperplastic tissue was grayish red and very friable. A phlegmonous inflammation extended to the right side.

The abdominal viscera were matted together with a frothy purulent exudate which almost encased the liver as a rather thin purulent looking membrane. The spleen was enlarged and friable. The lungs and kidneys were pale but mild acute purulent pleurisy without effusion was present.

Direct examination of the subcutaneous phlegmon and of the peritoneal exudate showed filamentous and fusiform-like organisms with numerous coccoid and gonidial forms. The picture was strikingly like that of the blood culture of the animal, which had numerous small cocci and gonidial forms with an occasional spirillary element. (Cf. figure 6 from the blood of a clinical case of gangrenous balanitis.)

Experiment 8. Blood culture of the pig of experiment 7 was incubated with organism X but was grown at room temperature. One cubic centimeter was inoculated subcutaneously into a 400-gram pig on April 17, 1918.

Results. The local induration that developed in twenty-four hours was without slough but had by April 21, 1918, developed a slough and extended into the inguinal region. The pig was obviously weak and toxic and had decreased in weight from 400 to 300 grams. Died.

Autopsy protocol. An extensive phlegmon spreading from the site of inoculation was associated with a regional and somewhat generalized lymphadenitis. The hemorrhagic lungs were encased in a purulent membrane which also enveloped the liver like a sac. Fresh adhesive pericarditis was evident. There was a generalized exudation throughout the abdominal cavity but without free fluid. Smears from various portions showed fusiform bacilli, filaments and cocci.

Pathologic summary. Discussion. There is a noteworthy similarity in the general pathology and the bacteriology of both these animals. The findings are of suggestive import in their possible relation to the origin of the clinical condition in human beings known as polyserositis. This has often been thought to be due to tuberculosis and the causative organism here is in its cultural aspects much like the tubercle bacillus except that it is not acid fast.

Especially noteworthy is the fact that we have with this organism a fusiform-filamentous complex associated with a coccus, much as we had in the case from which the organism was isolated. In common with it, too, there was no suggestion of motile spirochetes. Such a complex, when it becomes generalized as it occasionally does in the human being, runs a course which is strikingly like that of a generalized tuberculosis, particularly in respect to the alternate remissions and exacerbations that it shows.

Whether spirochetes are present may depend on which variety of filtrable gonidium gives rise to the pathologic process. The cultural work on these two animals was not extensive enough to know with certainty whether the spirals present had actually been dissociated as a stabilized stage in the life history of the thread form, or whether they represented an ontogenetic phase in its phylogeny.[2] The same may be said of the large number of theta bacillus forms of experiment 7. This bacillus is recognized as a member of the anaerobic gangrene group, closely related to *B. fusiformis*.

Of note, too, is the difference in virulence of filtrable gonidia produced in different ways. This can best be seen by contrasting the above experiment with experiment 9 which follows. In this experiment the gonidia were derived not by symbiosis with organism X but they occurred rather spontaneously, arising laterally and terminally as illustrated in figure 5 and described in our original communication (1919a) as gonidia of the second order. Myriads of them could be seen developing from the filaments which did not break up in toto into a mass of granules as was the case when grown with organism X.

Experiment 9. Filtrate IX represented a filtrate of vibriothrix gonidia which occurred spontaneously as noted above. Immediately part of the filtrate was seeded with organism X, while a part was permitted to germinate at 37°. This it appeared to do in twenty-four hours, yielding a turbid broth containing coccoids and granular diphtheroid or fusiform looking bacilli (fig. 7 at *a*); but curiously enough all attempts to transplant

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[2] For fuller discussion of these relationships involving Haeckel's Law the reader is referred to Study V of this series (see bibliography).

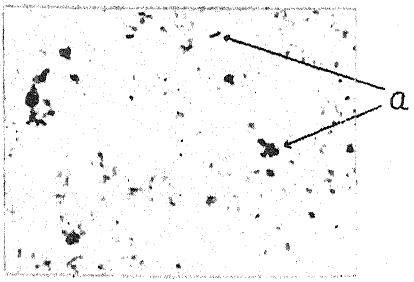

fig. 7

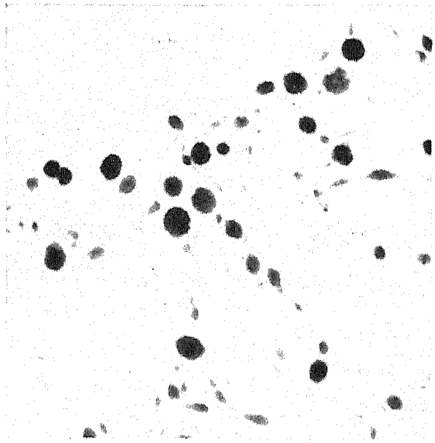

fig. 8

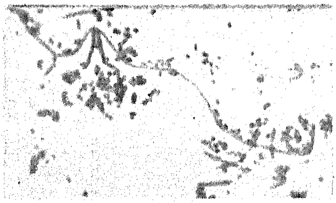

fig. 9

these forms were negative. These coccoid forms were grown with organism X as usual.

Results. The pig that received the ungerminated filtrate was quite negative in all respects after ten days' careful observation. The other one developed a rather innocuous lesion which suppurated nevertheless and gave myriads of diphtheroid and fusiform-like organisms in the pus. The lesion healed soon and gave no further trouble.

Experiment 10. The vibriothrix with giant coccoids (exospores figure 8)[3] but without visible gonidia was emulsified in broth to which was added a broth culture of organism X. Of this 0.5 cc. was injected subcutaneously into a pig.

Results. Only after a week was a slight suppuration observed and in the pus were found some long granular filaments and the solid type of *B. fusiformis.* The lesion was not progressive and healed rapidly with no further sequelae. Thus neither with adult organisms, the germinated gonidia nor the freshly filtered ones, do we get lesions that are at all comparable to those produced by gonidia of the diphtheroid or by those developed when the vibriothrix is grown symbiotically with organism X.

D. Results of filtration of clinical cases of infection with B. fusiformis and organisms other than isolated from case J. B.

Experiment 11. Diphtheroid bacillus J strain. This is a stabilized, relatively nonpleomorphic variant from the pleomorphic J strain described in our first communication on diphtheroids (1917). The organism was shown to have a nonhemolytic streptococcic phase that could be stabilized as such. It was quite granular and under certain conditions gave rise to numerous gonidial granules, as did the diphtheroid of the J. B. case.

These gonidia were emulsified in broth and filtered through a Berkefeld N. No growth was obtained under any conditions tried, but the filtrate was grown eighteen hours with organism X and injected into a 525-gram pig subcutaneously.

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[3] These photographs have a magnification of 1800 diameters while the other photographs have 1200.

Results. Within three days the injection site formed an ulcer which rapidly increased in size. Its margin became thickened and indurated and underneath there was a copious purulent exudate containing myriads of fusiforms, both granular and solid types. Many cocci and coccoids were present, the bipolar appearance of the latter probably representing young *B. fusiformis.* Spiral forms were occasionally present but they were not examined for motility. Large numbers of *B. thethoides* forms occurred (figs. 2 and 3).

Cultured aerobically the fusiforms did not grow and there was no odor to the cultures. On blood agar under anaerobic conditions numerous colonies of the elevated gray colonies of fusiform bacilli of solid type, mixed with cocci, were present (fig. 9). Some of these formed threads of varying length. The culture had the characteristic odor. The threads were Gram-negative. Later a few colonies of Gram-positive diphtheroids appeared which resembled the original culture.

Although the original J strain was a pure line one it occasionally showed a moderate filamentous change, the latter being Gram-negative and originating from the ends of the Gram-positive bacilli. As such they represented an ontogenetic phase in the culture's phylogeny. In the pig the thread forms became stabilized as developmental stages of *B. fusiformis.* This is but another example of a fundamental change that we have so frequently verified in vitro.

Experiment 12. Results with a long standing B. fusiformis infection of the lungs. This case—L. C.—proved to be one of the most interesting and clear cut cases of *B. fusiformis* infection that we ever studied. It was uncomplicated by the presence of spirochetes. The patient, eighteen years of age when we first observed her, had been a sufferer from a bronchial abscess since two years of age. The lesion followed whooping cough. She had been repeatedly diagnosed as tuberculous, but the organisms were never found in her sputum although they were searched for often enough by many observers, including the Saranac group. She had periodic attacks of fever and a high polymorphonuclear leucocytosis, 20,000 to 25,000. Search of her washed sputum showed *B. fusiformis* in purity, the colonies appearing grossly as little opaque cohesive flecks. The organisms were often in threads of moderate length.[4]

Filtrates of the sputum grown symbiotically with organism X, followed by pig inoculation, produced a small abscess containing curved mycelial and fusiform organisms, but the ulcer rapidly healed and the pig remained permanently well.

Three times the patient's blood was drawn into sterile broth or citrate solution. Part of it was seeded with the organism X and incubated at 37° for eighteen hours. Two of the three pigs developed the usual local lesions with *B. fusiformis* and filamentous forms. One died in two months and the other in two weeks, and at the autopsy marked serofibrinous adhesions and lung involvement were found. The same general flora was found as was present in the local abscess. The pig that was not affected received blood taken at a time when no febrile exacerbation existed.

Discussion and résumé. These results, apparently indicating the presence of a phase of *B. fusiformis* in the blood of seriously infected cases, are of interest because the fusiform organism is at best rarely isolated from the blood, even in cases of pyemia where one would suspect its presence. This may be explained by its occurrence in the filtrable form which, as we have seen, is not to be cultivated under the usual conditions. The progressive type of lesion developed from the blood culture suggests the presence of larger numbers of the gonidia there than in the sputum. From the direct examination of the sputum we could not be sure that gonidia were produced to any extent.

It is a matter of considerable interest that positive blood cultures in these cases when they occur usually yield a coccus; yet a careful examination almost always discloses the presence of filaments of whose origin from the coccus or its related coccoid forms there can be no doubt[5] (see fig. 6). It will be recalled that this was true for our original case (J. B.) and in experiments 7 and 8 was true for our experimental animals.

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[4] This patient was treated with potassium iodide and a hygienic régime. She later married and is perfectly well today.

[5] For more complete discussion and reports of blood culture findings in these cases reference may be made to our communication in 1919 (see bibliography).

The fact, too, that we have repeatedly shown in vitro that these diphtheroid organisms can dissociate stabilized coccus phases from their life cycle makes this relationship in vivo a more satisfactory explanation than the secondary infection theory. The single celled method shows clearly that the filaments as they occur in the cultures of the cocci are ontogenetic (pleomorphic) phases of the organism and not independent entities; but their presence always signifies that such a culture is *capable* of stabilizing its ontogenetic phase as a phylogenetic race or type. (See Study V.)

One of the clearest examples of the presence of these filaments as a purely pleomorphic phase of an otherwise typical coccus is shown in figure 6. This organism was repeatedly recovered from the blood of a patient with a severe gangrenous balanitis, the so-called fourth venereal disease. This condition is an example of Vincent's disease affecting the penis, and has the identical bacteriological diagnostic picture found in Vincent's angina. These cocci agglutinated the patient's serum in a 1:50 dilution, but a normal serum showed agglutination no higher than 1:10.

Experiment 13. A case of benign tumor of the large intestine (lipoma) so stretched the mucosa of the bowel as to give rise to superficial ulcerations of a very hemorrhagic character. The flora was of the fusospirillary type. The regional lymph glands were generally enlarged and presented an appearance suggestive of lymphosarcoma.

The patient developed a severe anemia, scarcely to be distinguished from pernicious anemia, with a lymphocytosis of 70 per cent. He later died with symptoms of progressive weakness and a general intoxication. The obscurity of the case and its relapsing character, combined with the uncertainty of a definite diagnosis, pointed to the probability that we dealt with a fusiform-streptothrix infection, secondary to a neoplasm that was essentially benign. It is of note that the chronic and relapsing type of the disease is exemplified in some of the experimental animals.

The lymph glands were ground up and filtered and grown with organism X, and when injected into a guinea pig yielded the same findings as gonidia from the other sources detailed—numerous fusiform and spirochete-like forms, cocci and diphtheroid forms. One of the two pigs so treated died after two weeks. There were no autopsy signs of note.

Experiment 14. This case was the filtrate from the throat of a typical ulcerous Vincent's angina. Treated as all the other filtrates were and inoculated, a similar sloughing ulcer developed with the typical flora noted before. No true spirochetes could be identified with certainty. Other cases, milder clinically, such as gingivitis associated with *B. fusiformis*, gave negative or equivocal results. We have not had adequate opportunity to apply the method to a sufficient number of cases of frank Vincent's angina to judge of its validity or to ascertain whether the infective gonidia are present only in the early stages of the disease. We have a suspicion that this is the case.

THE BEARING OF THESE EXPERIMENTS ON INFECTION AND EPIDEMIOLOGY

The bearing of these experiments in the direction suggested by the subtitle is obviously of import, although it is not possible to do more than allude to these considerations here. It is desirable to point out, however, that the study as a whole represents an application of the broad principle of polymorphism—in its true mycological sense—to infection. These infective gonidia are undoubtedly a form of seed or spore, even though they are not heat resistant. Their capacity only becomes manifest when the conditions for their germination are realized.

But when they germinate as the fusiform bacilli, or the thread form perhaps, they have already passed into a nonvirulent phase. This explains the difficulty with which experimental animals have been infected with organisms from this and similar cases. Epidemics of this infection in the human being have been explained on the *presence* of fusiform bacilli in normal mouths, which does not, of course, explain why epidemics of this—or any other conditions for that matter—are not always with us.

In these alternating cycles of virulence that can be correlated with the botanical characters of microörganisms we have something that helps us to understand the well known cadences of epidemic virulence shown by infectious diseases.

But of first importance is the fact that this botanical correlation may simultaneously involve a change in morphologic type—that is to say, a fusiform infection may actually take origin from a diphtheroid, the latter being the aerobic stage which, when "adapted" to anaerobic conditions, is a fusiform bacillus. We know, too, that the reverse of this change takes place as I indicated on page 490 of my original diphtheroid monograph (Mellon, 1917). In this instance a virulent anaerobic fusiform type of diphtheroid became quite avirulent when it was transformed to its aerobic diphtheroid stage. This organism was referred to as an anaerobic diphtheroid but its ends were distinctly pointed and it was slender and granular. Its aerobic stage was a different morphologic type, but serologically identical with the anaerobe.

Study III of this series (1926) shows an acquisition of marked virulence by a *B. alkaligenes* after it had changed from a bacillary to a streptothrix type of organism; yet the serologic characters had not been altered to any extent. Thus it appears that the acquisition of virulence by a filtrable phase of an organism is but one application of the broad principle of polymorphism; for there is no a priori reason why a filtrable organism should be virulent and the nonfiltrable avirulent. But we so frequently associate the terms "filtrability" and "virus" with virulence that unless we keep in mind the *principle*, we are in danger of using the terms as catchwords.

The recent studies of Vaudremer (1923) indicate that from the tubercle bacillus a mycelial stage can be dissociated in more or less stabilized form. Although non-acid fast it is capable of reverting to an acid fast tubercle bacillus but rather different from the parent form. The organism originates from the normal bacillus as a filtrable non-acid fast form which grows out as a gonidial producing mycelium. He believes it has protective value in guinea pigs.

Vaudremer's results emphasize the inadequacy of interpretation that has been placed on Metchnikoff's well known observation of branching forms for the tubercle bacillus. Its possible taxonomic bearing has been the only significance attributed to it, but the stabilization of such a stage which is different biologically gives Metchnikoff's observation its really significant bearing. Moreover, the *distinctive* taxonomic position which branching gave the tubercle bacilli shrinks into relative insignificance, since present day work indicates that it is not different in this respect from most other bacteria.

This work with the tubercle bacillus parallels our own study with the fusiform bacillus, showing as it does that a *complex* of so-called distinct types really exists in each instance. The growing tendency to place the *B. fusiformis* near the streptothrices is borne out, *in a sense*, by showing experimentally that like the tubercle bacillus it has such a stage in its life history. Furthermore the genetic relations of *B. fusiformis* to other organisms of the anaerobic gangrene group is indicated, for in the lesions of the animals the theta bacillus developed abundantly—as a stabilized stage or a phase—it matters not which, since the potentialities are the same.

Clinically we frequently see cases with mouth lesions resembling a Vincent's angina, but not typical. We are usually able to isolate an organism, fusiform-*like* perhaps, or it may be the theta bacillus. The lesions we regard as a mild Vincent's and usually they run such a course. They do not tend to recur as does the latter. Great differences in respect of chronicity and relapse were noted in our experimental animals.

Since our ordinary bacteria have filtrable phases we are now in a position to ask legitimately whether some of our so-called viruses are not the stabilized stages of our visible bacteria. It appears that these filtrable stages of bacteria cannot be sharply demarcated from a virus. They may both be invisible, extremely difficult of cultivation and more resistant to certain deleterious influences than the vegetative forms of the bacteria. Even the supposedly distinctive resistance of the true viruses to 50 per cent glycerine appears to have been broken down by Miss Evans who has cultivated the specific green streptococcus of encephalitis from these glycerinized emulsions!

So this is another point where the prevailing distinctions in biologic life may not be valid in the light of the polymorphism of the mycologist. The importance of this point of view has obvious applications, e.g., in the genetic relations of the proteus X-19 to the Ricksettia of typhus fever, a view which is finding an increasing number of proponents among those whose investigations entitles them to an opinion.

A similar situation may exist between the virus of hog cholera and *B. suipestifer*, or between a virus of epidemic virulence in guinea pigs that is constantly associated with a hog cholera-like bacillus (Petri and O'Brien, 1910); and we now know that the typhoid bacillus has a virulent filtrable phase, not ordinarily cultivable, that produces the animal picture and the serologic reactions of typhoid as faithfully as do injections of the visible organism (Friedberger, 1923).

Where a specific type of visible organism is constantly associated with a disease whose existing agent is a virus, the prevailing notion that the visible organism is an unrelated secondary invader will, I fancy, require a degree of modification that will fit more closely the increasing number of facts. A coccus consistently found in the blood stream of patients with an apparent streptothrix or *B. fusiformis* infection is in principle not different from the situations just cited.

Nor is this situation fundamentally different from that of the human disease of blastomycosis. Studies VI and VII of this series (Mellon, 1926) show beyond reasonable doubt that the polymorphism of these fungi finds expressions in vivo and in vitro by a dissociated complex of stabilized types that have proved a stumbling block for the monomorphic etiology of infectious diseases; for even the most reactionary bacteriologist cannot but acknowledge that the blastomycetes of this disease have a real life cycle. The experimental pathogenesis of blastomycosis is notoriously obscure and the probabilities are that the actual infectious phase of this fungus complex that we conventionally isolate is as unknown to us as have been the infective gonidia of *B. fusiformis*.

It may be indeed as Friedberger suggests that the initial infecting stage of all microbic diseases is an invisible uncultivable virus *phase*, and the organisms that we isolate are as often as not the avirulent stage. In this connection it is interesting to recall the classic experiment of Pettenkofer who in the cholera epidemic of 1892 drank pure cultures of the *B. cholera* cultivated from the dejecta of patients, but with negative results; while those drinking water from which no *B. cholera* bacillus could be isolated came down with the disease. Such a paradox may be linked up with the ancient view of those doubting Thomases who believed that the *disease produced the bacteria*, and not bacteria the disease!

However this may be, it is becoming increasingly difficult to dissociate entirely the saprophytes—so-called—of nature from the parasites of living things. The *thinking* epidemiologist is now admitting this point of view to his consideration as evidenced by the excellent paper of Richard Strong read before the Association for the Advancement of Science in Washington two years ago.

CONCLUSIONS

1. Evidence has been adduced that *B. fusiformis* and related organisms often associated with it in infective processes have a filtrable or gonidial phase on which their virulence primarily depends—an infective granule, so to speak.
2. This phase is usually not to be cultivated under the same conditions that enables the parent form to germinate. Some of these forms are probably invisible.
3. The conditions that are suitable for the germination of this phase, particularly in vivo, may result in its cyclic evolution to a totally different stage (bacterial type) in the infectious complex.
4. The various members of this complex, or part of them, may be avirulent in their adult phase, but quite virulent in the filtrable phase.
5. Different orders of these gonidia seem to differ greatly in virulence. Some may be of lesser virulence than the parent form of the organism.

6. The fact that common nonpathogenic organisms may have a virulent, filtrable, but uncultivable phase in their life history, suggests a revaluation in our conception of the viruses, so-called.

7. It also suggests a revaluation in our views as to what constitutes a secondary infection.

8. The latter are obviously of two kinds: *First*, those genetically related to the infective or filtrable form and helping to make up the infective complex, and *second*, chance invaders having no genetic relation to any member of the complex.

9. Such considerations have an obvious and important bearing on current views of epidemiology, infection and immunity.

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NOTES AND QUERIES

Material for this column should be sent to C.-E.A. Winslow, Yale School of Medicine, New Haven, Conn., and will be given publication as promptly as circumstances permit. In answering queries please note the number of the query and the issue of the JOURNAL to which the answer applies. If replies are made directly to the author of a question, it is requested that a copy of the reply be sent to the editor of the JOURNAL.

9. Replying to query No. 1, Jour. Bact., **12**, 85:

The undersigned has published evidence on the bacterial nature of mitochondria (Am. Jour. Anat., Vol. 30, Vol. 33, Vol. 35, Vol. 36; Am. Nat., Vol. 57; Anat. Rec., Vol. 26). A resumé of the evidence, together with an hypothesis on the fundamental significance of mitochondria is being published in book form by The Williams & Wilkins Company of Baltimore.

IVAN E. WALLIN.

Denver, July 27, 1926.

10. Replying to query No. 3, Jour. Bact., **12**, 85:

Under separate cover I have mailed a petri dish containing two giant colonies of red (?) yeasts; their source being creamery butter plated on wort agar. This culture is about one year old, and its color appears to be nearer the blood red than the salmon pink.

PAUL L. SCHWARTZ

11701 95 A Street, Edmonton, Alberta, Canada.

11. In a paper by Dr. Ivan C. Hall, in the June number of the JOURNAL OF BACTERIOLOGY, the impression is given that the anaerobic cultures carried in the American Type Culture Collection are the same as those kept at the American Museum of Natural History in New York City in 1922. As a matter of fact none of these cultures of anaerobes were among those transferred to Chicago from Washington. Of the cultures sent from New York to Washington only a few of the original ones reached Chicago, and those were nonpathogenic aerobic sporulating bacteria.

The anaerobic cultures now kept in the Collection and sent out upon request have been secured from I. C. Hall, K. F. Meyer, G. F. Reddish, and Carl Ten Broeck. A few have come from the Hygienic Laboratory in Washington and E. M. Houghton, Parke Davis & Co., Detroit. In the case of these, as all cultures, the Committee in charge of the American Type Culture Collection endeavors to secure authentic cultures from reliable sources and every effort is made to preserve cultures in purity. In connection with a list of available cultures issued by the Committee last year, it is stated "Every effort is made to maintain in a state of purity cultures of known authenticity, but the Committee can not guarantee that cultures distributed are free from contamination, or true to description. Prompt report of evidence of contamination or failure to conform to accepted descriptions, and constructive criticism of the service is requested." This implies that everyone who begins work with a culture which comes to him from any source will first assume himself of its purity and identity, and is in full accord with the statement by Dr. Hall as to the "first duties of any author, etc."

I hope you may find a way to put this matter before the readers of the JOURNAL, as it is of great importance in the usefulness of the Culture Collection.

GEORGE H. WEAVER, *Curator,*
American Type Culture Collection,
John McCormick Institute for
Infectious Diseases
637 South Wood Street, Chicago, Ill.

PRELIMINARY ANNOUNCEMENT OF THE FIRST INTERNATIONAL CONGRESS OF SOIL SCIENCE

In accordance with the decision of the Fourth International Conference of Soil Science that met in Rome, in May, 1924, the First Congress of the International Association of Soil Science, then organized, will convene on June 13, 1927, in Washington, D. C. The Congress will be followed by a field excursion to visit the various important soil belts in the country. Opportunity will also be given to the delegates to acquaint themselves with various agricultural industries, some of the leading Agricultural Experiment Stations, and in general with the agricultural resources of the United States.

The Association is made up of the following six International Commissions:

I. Soil Physics. Chairman, Dr. V. Novak, Czechoslovakia; American representative, Dr. C. Davis, Bureau of Soils, Washington.

II. Soil Chemistry. Chairman, Prof. A. de Sigmond, Hungary; American representative, Dr. M. M. McCool, East Lansing, Mich.

III. Soil Bacteriology. Chairman, Prof. J. Stocklasa, Czechoslovakia; American representative, Dr. S. A. Waksman, New Brunswick, N. J.

IV. Soil Fertility. Chairman, Prof. E. A. Mitscherlich, Germany; American representative, Prof. D. R. Hoagland, Berkeley, Calif.

V. Nomenclature, Classification and Cartography. Chairman, Prof. C. F. Marbut, U. S. A.

VI. Application of Soil Science to Land Cultivation. Chairman, Dr. J. Girsberger, Switzerland; American representative, Dr. S. H. McCrory, Bureau of Agricultural Engineering, Washington.

Each Commission is now working on the preparation of its own program. Some of the sessions will be devoted to the Congress as a whole or to combined meetings of more than one Commission, while a number of sessions (5 to 8) will be devoted to the special sessions of each Commission.

The program of each Commission will consist of papers presented by invitation by outstanding investigators in the respective fields, and of papers presented by various workers in the different branches of Soil Science, by members or non-members of the Association. Titles of the papers to be presented and brief abstracts in English, French and German should be sent on or before December 1st, either to the respective Chairman, or to the American Representative of the Commission, where the paper is to be presented, or to the President of the Association, who will have the paper forwarded to the Chairman of the corresponding Commission.

This Congress will bring together in this country, for the first time in its history, all those that are interested in the different problems of soil classification, soil analysis, fertilization and treatment, as well as the relation of the soil to plant growth. Extensive exhibits of various soil types (monolithic columns, in respective horizons) from Europe and America, apparatus used in soil analyses, of the soil microflora and microfauna, etc. will be held during the Congress.

Dr. J. G. Lipman,
President International Society
Soil Science, New Brunswick,
N. J.
Dr. D. J. Hissink,
Secretary, Groningen, Holland.

RELATION BETWEEN THE CHEMICAL CONSTITUTION AND GERMICIDAL ACTIVITY OF THE MONOHYDRIC ALCOHOLS AND PHENOLS

F. W. TILLEY AND J. M. SCHAFFER

From the Biochemic Division, Bureau of Animal Industry, United States Department of Agriculture

Received for publication January 1, 1926

This paper presents the results of bacteriological tests of the germicidal activity of a considerable number of monohydric alcohols and phenols, made as a part of a systematic study of the relation between the chemical and physical properties and the germicidal activity of various classes of chemical compounds. It is expected that a detailed account of the studies of the chemical and physical properties, especially with regard to measurements of surface tension, will appear at a later date.

Many of the compounds studied were obtained from commercial sources. Those which could not be thus obtained were made in these laboratories. Whatever the source, however, all of the substances were very carefully purified. The boiling point and melting point, or solidification point, of each of the purified compounds is shown in table 1. The boiling point was determined by means of standardized Anschutz thermometers the stems of which were immersed in the vapors of the liquid during distillation. The table of Young (1902) was used in correcting boiling points for changes in barometric pressure.

BACTERIOLOGICAL WORK

The bacteriological tests have been made by the Rideal-Walker method (1913) modified as follows: First, by the use of the unadjusted culture medium devised by J. H. Wright (1917), made with Armour's "Peptonum Siccum" and having a pH value of approximately 6.5; second, by the use of a medication tempera-

TABLE 1

Physical constants of the substances tested

SUBSTANCE	FORMULA	BOILING POINT	MELTING POINT	SOLIDIFICATION POINT
		°C.	°C.	°C.
Methyl alcohol	CH_3OH	65.1–65.4		
Ethyl alcohol	CH_3CH_2OH	78.2		
Normal propyl alcohol	$CH_3CH_2CH_2OH$	97.1		
Normal butyl alcohol	$CH_3(CH_2)_2CH_2OH$	117.9		
Normal amyl alcohol	$CH_3(CH_2)_3CH_2OH$	137.2–138.0		
Normal hexyl alcohol	$CH_3(CH_2)_4CH_2OH$	156.6–158.5		
Normal heptyl alcohol	$CH_3(CH_2)_5CH_2OH$	175.3–176.3		
Normal octyl alcohol	$CH_3(CH_2)_6CH_2OH$	195.2–195.3		
Secondary propyl alcohol (dimethyl carbinol)	$CH_3CHOHCH_3$	82.2–83.0		
Secondary butyl alcohol (methyl ethyl carbinol)	$CH_3CH_2CHOHCH_3$	99.2–100.0		
Secondary amyl alcohol (methyl propyl carbinol)	$CH_3CH_2CH_2CHOHCH_3$	118.2–120.2		
Secondary hexyl alcohol (methyl butyl carbinol)	$CH_3(CH_2)_3CHOHCH_3$	138.0–140.0		
Tertiary butyl alcohol (trimethyl carbinol)	$CH_3COH(CH_3)_2$	82.2–82.4		
Tertiary amyl alcohol (dimethyl ethyl carbinol)	$CH_3CH_2COH(CH_3)_2$	102.0–102.2		
Tertiary hexyl alcohol (dimethyl propyl carbinol)	$CH_3CH_2CH_2COH(CH_3)_2$	122.6		
Phenol	C_6H_5OH	181.7		40.7
Para-cresol	$CH_3C_6H_4OH$	202.0		34.0
Para-ethyl phenol	$CH_3CH_2C_6H_4OH$	216.0–218.0		38.0
Para-propyl phenol	$CH_3CH_2CH_2C_6H_4OH$	231.7–232.3	18.2	
Para-butyl phenol	$CH_3CH_2CH_2CH_2C_6H_4OH$	247.5–248.5	17.0	

ture of 25°C.; third, by the use of *Staphylococcus aureus* as a test organism in addition to *Bacillus typhosus;* fourth, by making up test dilutions in sterile Erlenmeyer flasks instead of stoppered cylinders, adding fixed amounts of stock solution to the varying amounts of sterile distilled water required, transfers of stock solution and water being made with sterile standardized pipettes; fifth, by using a 1 cc. pipette graduated into tenths instead of a dropping pipette for measuring the 0.5 cc. of culture added to each 5 cc. tube of test dilution; and sixth, by deducing coefficients at any exposure time after five minutes instead of only after seven and one-half minutes.

It is important to note that the original stock solutions from which the test dilutions were derived were in all cases made up so as to contain a definite weight of disinfectant in 100 cc. of solution. The stock solution of phenol used in preparing the phenol standard tubes contained 5 grams of phenol per 100 cc. Other stock solutions, especially those of the lower alcohols, were made up so that their concentration was as near as possible to that of the test dilutions in order to minimize possible errors due to changes in volume on dilution with water. Each coefficient, then, is based on the relative amounts by weight of the disinfectant and of the phenol standard required to kill the test organism in the same time, whereas the usual procedure is to compare dilutions of the phenol standard made up by weight with dilutions of the disinfectant made up by volume.

In order to obtain greater accuracy, three or more tests were made on each sample, and each coefficient shown in the tables is the average figure obtained from three or more closely concordant results.

In addition to the ordinary phenol coefficients there are shown in the tables coefficients which were based on equal molecular concentrations, these being designated "molecular" coefficients. These were derived from the phenol coefficients by the use of the following formula:

$$\frac{\text{Molecular weight substance tested}}{\text{Molecular weight of phenol}} \times \begin{matrix}\text{phenol}\\ \text{coefficient}\end{matrix} = \begin{matrix}\text{molecular}\\ \text{coefficient}\end{matrix}$$

TABLE 2

Coefficients of primary normal alcohols

NAME	PHENOL COEFFICIENTS	MOLECULAR COEFFICIENTS		
		Observed	Ratios	Calculated
1. Test organism *B. typhosus*				
Methyl	0.026	0.009	2.2	
Ethyl	0.040	0.020	3.25	
Propyl	0.102	0.065	3.30	0.067
Butyl	0.273	0.215	3.39	0.225
Amyl	0.78	0.73	3.39	0.75
Hexyl	2.3	2.5	3.38	2.52
Heptyl	6.8	8.4	3.46	8.46
Octyl	21.0	29.0		28.2
2. Test organism *Staph. aureus*				
Methyl	0.030	0.010	1.9	
Ethyl	0.039	0.019	2.8	
Propyl	0.082	0.053	3.30	
Butyl	0.22	0.175	3.37	0.178
Amyl	0.63	0.59		0.598

TABLE 3

Coefficients of secondary and tertiary normal alcohols

	TEST ORGANISM B. TYPHOSUS			TEST ORGANISM STAPH. AUREUS		
	Phenol coefficients	Molecular coefficients	Ratios	Phenol coefficients	Molecular coefficients	Ratios
Secondary alcohols:						
Propyl	0.064	0.041	2.9	0.054	0.034	3.0
Butyl	0.152	0.120	3.0	0.131	0.103	2.9
Amyl	0.38	0.36	3.0	0.32	0.30	
Hexyl	1.00	1.09				
Tertiary alcohols:						
Butyl	0.081	0.064	2.7	0.064	0.050	2.7
Amyl	0.182	0.170	2.9	0.142	0.133	
Hexyl	0.45	0.49				

TABLE 4

Coefficients of the para-phenols

NAME	TEST ORGANISM B. TYPHOSUS			TEST ORGANISM STAPH. AUREUS		
	Phenol coefficients	Molecular coefficients	Ratios	Phenol coefficients	Molecular coefficients	Ratios
Para-cresol	2.5	2.9	3.3	2.2	2.5	3.1
Para-ethyl phenol	7.4	9.6	3.2	6.0	7.8	3.1
Para-propyl phenol	21.6	31.0	3.5	16.5	24.0	3.3
Para-butyl phenol	68.0	108.0		50.0	79.0	

The above-mentioned coefficients for the primary, secondary and tertiary normal alcohols and para-phenols are shown in tables 2, 3 and 4. Examination of the tables shows that there is an approximately constant ratio between the molecular coefficients of successive members of the same series, so that these coefficients are almost in geometrical progression. The ratios are not, however, absolutely constant, and there seems to be a tendency for the ratio between successive members of the same series to increase as we go upward in the series. The significance of this apparent tendency for the ratios to increase is as yet undetermined.

It will be noted in table 2 that if methyl alcohol be omitted the average ratio between the coefficients of the primary normal alcohols is 3.36. In view of the fact that methyl alcohol differs from the other members of the series in many of its properties, it is not surprising to find that its bactericidal power does not conform to a rule which applies to other members of the series. This average ratio 3.36 may be used in calculating the coefficients of higher members of the series from those of lower members with a considerable degree of accuracy. In this way the coefficients of the higher members of the series have been calculated from the coefficient of ethyl alcohol, with *B. typhosus* as the test organism, and from the coefficient of propyl alcohol, with *Staph. aureus* as the test organism. The coefficients thus obtained are shown in table 2 in the column headed "Calculated." When *Staph. aureus* was used as the test organism the observed ratio between the coefficients of ethyl and propyl alcohols was anomalous, and for that reason it was necessary to begin calculation with the ratio 3.36 from the coefficient of propyl alcohol as a starting point.

The phenol coefficients, molecular coefficients and ratios for the secondary and tertiary normal alcohols are shown in table 3 and those for the para-phenols are shown in table 4. The number of compounds tested is too small to make it possible to obtain the average ratio for each series with as great a degree of accuracy as with the primary normal alcohols, but the approximate ratios seem to be 3.0 for the coefficients of the secondary normal alcohols, 2.7 for those of the tertiary normal alcohols and 3.3 for those of the para-phenols.

DISCUSSION

It has been recognized by various authors that the germicidal activity of alcohols and phenols increases with increasing molecular weight (Rideal (1921), Morgan and Cooper (1912), Schaeffer (1917), Johnson and Lane (1921)), but, as far as the writers are aware, no one up to the present time has observed definite numerical ratios such as are reported in the present paper. However, Kamm (1921) has reported a similar relationship for the toxic action of the primary alcohols on Paramecia, the toxicity of any given member of the series being three times that of the preceding member when calculated on a molecular basis.

It is interesting to note that although the germicidal values of the normal alcohols increase regularly for each additional methyl group in the straight chain, a comparison of the isomeric alcohols shows a regular decrease in germicidal value from primary to secondary to tertiary. The relation between these germicidal values of the isomeric alcohols may be expressed very nearly by the proportion, primary is to secondary as secondary is to tertiary.

Unlimited increase in germicidal activity with increasing molecular weight is prevented by the decrease in solubility which also accompanies increasing molecular weight. As a result a point is reached finally where it is impossible to get enough of the disinfectant in solution to kill the test organism within the time limits of the method of testing employed. For example, with *Staph. aureus* as the test organism the coefficients shown in tables 2 and 3 are less in number than those for *B. typhosus* because solubility decreased to the point where it was impossible to get enough material in solution to kill *Staph. aureus*.

SUMMARY

Phenol coefficients have been determined for a number of primary, secondary and tertiary normal alcohols and para-phenols. When these coefficients are converted from the original gram weight basis to a gram molecular basis the coefficients of successive members of each series are found to increase almost uniformly, with an average ratio between coefficients of 3.36 for the primary normal alcohols and of approximately 3.0, 2.7 and 3.3 for the secondary normal alcohols, tertiary normal alcohols and para-phenols, respectively.

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THE CONCENTRATION OF SEA-WATER AS AFFECTING ITS BACTERIAL POPULATION

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In connection with certain studies on the relation of marine bacteria to the precipitation of $CaCO_3$ in the sea, carried out by the author at the Tortugas Laboratory of the Carnegie Institution of Washington in 1922, a brief study was made of the influence of dilution of sea-water on its bacterial population. The facts obtained in this study appear to be of sufficient interest to warrant their publication and to stimulate further study of the subject. They are, therefore, set forth below.

In the first part of this study, I determined the influence of dilution on the mixed flora of the sea-water occurring near the wharf of the laboratory where, of course, there is much contamination from shore flora. The sea-water just described was plated on four different media, made up as follows:

No. 1. One gram calcium lactate, 1 gram peptone, 0.5 gram potassium nitrate, 1000 cc. sea-water.

No. 2. Same as no. 1, but water supplied was made up of 750 cc. sea-water and 250 cc. distilled water.

No. 3. Same as no. 1, but water supplied was made up of 500 cc. sea-water and 500 cc. distilled water.

No. 4. Same as no. 1, but water supplied was made up of 250 cc. sea-water and 750 cc. distilled water.

Appropriate dilutions were made for the platings and after four days' incubation at laboratory temperature (approximately 28°C.), the final counts were made with the following results:

	Number of bacteria per cubic centimeter sea-water
Medium 1	960,000
Medium 2	730,000
Medium 3	640,000
Medium 4	680,000

On medium 1, sea-water from 1 mile out to sea showed only 100 to 200 bacteria per cubic centimeter.

It is clear from the foregoing data that dilution of sea-water close to shore, or what should amount to the same thing, reduction of the salt content of the medium on which it is plated, does result in depressing the numbers of organisms, the more so as sea-water is more and more diluted until equal proportions of sea-water and distilled water are employed. Still greater dilutions beyond that point, however, do not greatly depress the total number of organisms as judged by a bacterial count. It occurred to me, however, that this may be only a partial truth, inasmuch as certain organisms belonging normally to the shore would have greater opportunities for development with increasing dilution of the medium, whereas others belonging normally to uncontaminated sea-water would be depressed thereby. For that reason, I determined to study the influence of dilution of a sea-water medium like that above described on the growth of a pure culture of a sea-water bacterium found in pure ocean water as a normal inhabitant in tropical seas. For that purpose, I chose an organism which has been variously named (in my opinion, incorrectly) *B. calcis* (Drew), and *Ps. calcis* (Kellerman and Smith). On certain kinds of media, this normal marine bacterium, in common with other normally occurring bacteria in tropical sea-water, precipitates $CaCO_3$ from the medium. Other studies on this organism have been published elsewhere by the author[1] and a further communication on the subject will soon be forthcoming.

Five different media were employed in plating out the pure culture of the organism in question. They were as follows:

No. 1. Two grams calcium lactate, 1 gram peptone, 0.5 gram potassium nitrate, 1000 cc. filtered sea-water.

No. 2. Same as no. 1, but with $\frac{7}{10}$ sea-water and $\frac{3}{10}$ distilled water, making up the 1000 cc. of water used.

No. 3. Same as no. 1 but with $\frac{5}{10}$ sea-water and $\frac{5}{10}$ distilled water.

No. 4. Same as no. 1, but with $\frac{3}{10}$ sea-water and $\frac{7}{10}$ distilled water.

No. 5. Same as no. 1 but with $\frac{1}{10}$ sea-water and $\frac{9}{10}$ distilled water.

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[1] Publication 340, Carnegie Inst. of Wash., pp. 179–191.

Appropriate dilutions were made in all the platings but the counts were rendered easy on the second dilution of the suspension, namely 1:10. The results of the counts which represent averages of very closely agreeing replicates were as follows:

	Number of colonies from a given amount of suspension
Medium 1	270
Medium 2	200
Medium 3	150
Medium 4	None
Medium 5	None

The foregoing data seem to furnish unequivocal evidence of the effect of dilution of the sea-water medium on the ability of a typical marine bacterium to form colonies on suitable artificial media. The dilution of sea-water to the point of cutting its concentration into two seems to result in a depressing effect on the power of the organism in question to develop on the plates, but the depressing effect grows gradually; further dilution of the sea-water, in accordance with the scheme given above, proves to be completely destructive to the cells. Moreover, the appearance of the colonies on each medium described above is quite characteristic. On medium 1, there is a preponderance of large surface colonies, but also many deep colonies appearing as opaque dots at the bottom of the medium. On medium 2, there are very few surface colonies, and no large ones, but numerous deep colonies with denser nuclei than those appearing in the colonies of medium 1. On medium 3, there are no surface colonies, and the deep colonies are all very small, appearing as dense dots deep in the medium. On the other media, as indicated above, there were no colonies.

THE BACTERIAL FLORA OF SERPENTINE SOILS

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In collaboration with A. Gordon, the author has carried out a series of experiments described elsewhere[1] to determine the cause, or causes, of the infertility or virtual sterility of serpentine or, more generally speaking, of dominantly magnesian soils. In the course of those investigations, it was discovered that the soils in question are unsuitable plant media, not because of their high content of magnesium, but because of the high pH of the soil and its solution, and because of the deficiency of the latter in certain essential ions, and especially $-NO_3$ and $-PO_4$ ions. The marked deficiency in these ions, and especially the former, suggested the idea that interesting and unusual conditions might be found on a bacteriological study of such soils. Accordingly, a brief study was undertaken of three serpentine soils from the Mount Diablo region in California. The three soils were as follows:

No. 1. From the top of a serpentine hill, on which the rock was fairly well disintegrated and soil formed. A sparse and stunted growth of grass was present.

No. 2. From the top of a serpentine hill a few hundred yards from no. 1, on which the rock was only slightly transformed into soil. Very little vegetation, even of the stunted variety.

No. 3. From a little vale between hills no. 1 and no. 2. A well-formed soil, deep brown to black in color. Has the appearance of containing much organic matter, and looks fertile, but vegetation is not vigorous-looking, though better than that described for the hilltops.

These three soils were subjected to study for bacterial counts and for nitrifying and nitrogen-fixing flora. The very striking disparity between the results obtained and those usually obtained on any ordinary soil, constitutes a legitimate excuse for their presentation here. The several tests will be discussed separately.

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[1] Soon to appear in print.

THE BACTERIAL COUNTS

The medium used for plating was a soil extract agar made up from a soil extract of soil 3 and the necessary agar. The soil extract was obtained by boiling, for one-half hour, one part of soil with two parts of distilled water. Proper dilutions were made in plating by the use of sterile water blanks, made up with the same soil extract used in preparing the medium. The data for the counts as thus obtained are given in table 1.

TABLE 1

Bacterial counts on serpentine soils

DILUTION	SOIL 1	SOIL 2	SOIL 3
1:100	1,420	160	3000
1:1,000	170	13	800
1:10,000	8	11	71
1:100,000	0	0	8

A student of soils who is even slightly acquainted with soil flora can see at once in the foregoing data a striking exception to the general rule. The highest count, based on the figures for the first dilution, shows only 300,000 organisms per gram of soil, and that is the best of the three soils by the criterion mentioned above. In soil 2, the count shows only 16,000 organisms per gram of soil, and in soil 1, only 142,000. When the data for the other dilutions than that of 1:100 are used for appraisal, nothing different in principle results. There is a striking paucity in the microorganic population in serpentine soils which must be referable to the chemical nature of the soil mass. Moreover, it is clear from the foregoing data that there is some dominant inhibiting factor common to serpentine soils which limits bacterial development, the three soils being widely different in many respects.

A study was made of the colonies developing on the plates employed for the counts, the results of which are set forth below for each soil separately.

Soil 1. Seven different kinds of bacterial colonies, three fungus colonies, and one *Actinomyces* colony, were found. Of the seven bacterial forms, one was *Sarcina lutea*, one was an ochre colored colony, and five were white to cream-white in color. The colonies varied from 1 to 2 mm. in diameter. Of the fungus colonies, one appeared to be a *Penicillium* and the other two were probably *Fusarium* forms. The one *Actinomyces* species was not identified. There were thus very few chromogenic forms, and bacteria predominated.

Soil 2. Six different kinds of bacterial colonies were found. No fungi or *Actinomyces* were observed. All the bacterial colonies were white and approximately 3 mm. in diameter or in extent. The forms of the colonies varied from round to star-shaped.

Soil 3. Six different kinds of bacterial colonies, and three *Actinomyces* colonies were found. No fungi. All the bacterial colonies were white and all except one were opaque. They varied from 1.5 to 3 mm. in diameter. Their shape varied from round to lenticular. The *Actinomyces* colonies were about 2 mm. in diameter, and dense; white to gray in color.

It will be observed that there is little diversity, as well as a general paucity, in the bacterial flora of the serpentine soils. Chromogenic forms are conspicuous by their total absence in two of the three soils, and almost absent in the third. Fungus and Actinomyces forms are also conspicuous by their absence or rarity.

NITRIFYING AND NITROGEN-FIXING BACTERIA

A test was made of the ability of the serpentine soils to nitrify ammonia or nitrite in the usual nitrification solutions. No nitrification was noted with any one of the soils even after two months of incubation. Some cells were observed in the cultures which resembled *Nitrosomonas*, but the observation may have been in error as regards the identification of the form as the cells were physiologically inactive. We thus have the very unusual experience of finding soils which possess no nitrifying power. In the writer's experience with hundreds of tests on nitrification with as many soils, this is the first instance of soils free from excess of salts or toxins, which contained no nitrifying bacteria, or at least, in which the nitrifying bacteria, if present, were wholly inactive.

In examining the same soils for *Azotobacter*, we found only a few cells in soil 1, but none in soil 2, nor in soil 3.

It would, therefore, appear to be true in serpentine soils that, because of the paucity in bacterial flora, and because of the small amount of ammonifiable nitrogen, the nitrifying bacteria cannot maintain themselves. Likewise because of the lack of sufficient readily decomposable and soluble carbon compounds, *Azotobacter* forms cannot live or function in these soils. The high pH which Gordon and the writer have found to be characteristic of these soils is not responsible for inhibiting the nitrifying bacteria from developing, since Meek and the writer[2] proved them to be resistant to much higher pH values. Others have found the same to be true. Likewise, *Azotobacter* has been found in the writer's experiments and in those of other investigators to flourish in a pH much greater than that found in our serpentine soils. The deficiency of the soil solution in $-PO_4$ ion may, however, operate to depress or inhibit the development of both the nitrifying bacteria and *Azotobacter*.

SUMMARY

1. Serpentine soils contain a very small microorganic population. This is true for very diverse serpentine soils.
2. The population found consists mostly of non-chromogenic bacterial forms. Very few fungi and *Actinomyces* are found.
3. No nitrifying bacteria exist, or at least none function in any of the soils studied.
4. A few *Azotobacter* cells were found in only one of the three soils.
5. These strikingly impoverished conditions of microorganic population in the serpentine soils are attributed to (1) deficient ammonia supply, in turn owing to deficiency in readily decomposable proteins and similar substances, (2) paucity in ammonia producing microorganisms, (3) deficiency in available $-PO_4$ ion, and (4) deficiency in carbon compounds.

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[2] Jour. Gen. Physiol., November 20, 1922, 5, 2, 195–204.

A METHOD FOR THE STUDY OF THE FORMATION OF VOLATILE SULFUR COMPOUNDS BY BACTERIA

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The study of hydrogen sulfide formation by bacteria has long been handicapped by the lack of an accurate method for the quantitative estimation of very small quantities of the gas. The lead acetate paper test may be used where quantitative accuracy is of little importance, but this method is too crude for exact determination. Lead and iron salts have been added directly to the culture media by various investigators (Wilson, 1923) as a qualitative and approximately quantitative test for differentiation purposes.

Recently the determination of hydrogen sulfide by titration with standard iodine solution has been advocated. Heap and Cadness (1924) employed N/100 solutions of iodine, a stream of carbon dioxide being used to carry over the hydrogen sulfide. Fellers, Shostrom, and Clark (1924) recommend N/40 iodine solutions and separation of the hydrogen sulfide by means of a current of air. These methods are open to the objection that they are not sufficiently delicate and are not specific for hydrogen sulfide, as other substances may be oxidized by the iodine (mercaptans).

With a method recently devised (Almy, 1925), hydrogen sulfide in quantities as small as 0.002 mgm. (2 micromilligrams) may be satisfactorily determined. The new procedure has the added advantage that, so far as we have been able to determine, it gives hydrogen sulfide only. The determination is accomplished by aeration of the acidified aqueous suspension or solution of the samples with carbon dioxide, absorption of the evolved hydrogen sulfide in dilute zinc acetate solution, and evaluation of the hydrogen sulfide by a measurement of the strength of "methylene blue" color produced upon adding p-amino dimethylaniline hydrochloride, hydrochloric acid, and ferric chloride to the zinc acetate mixture. The original article gives the details of the method.

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[1] The senior author is responsible for the chemical part and the junior author for the bacteriological part of this paper.

This methylene blue method was successfully adapted to the study of hydrogen sulfide formation by bacteria in broth cultures. Certain features were considered essential for the proper conduct of such studies. It should be possible to follow the *rate* of formation of hydrogen sulfide without loss of the gas, without changing the conditions of growth of the bacteria and with the oxygen supply under adequate control. Following the rate by withdrawal of aliquot portions of the broth for analysis is obviously of no value, as no account is taken of that not inconsiderable portion of the gas which escapes from the medium. This loss would naturally be greater while the medium was acid than while it was alkaline. Likewise it is not satisfactory to attempt to follow the rate by intermittent aeration of a single broth culture, because not all of the gas would be removed in this manner when the medium is alkaline. Furthermore, the irregular aeration would introduce a variation in the condition of the medium which would not be comparable with the conditions obtaining in ordinary cultures, which usually remain undisturbed during the entire incubation period.

PROCEDURE

Bearing these matters in mind, the following apparatus and procedure were devised. The medium is sterilized in a 2-liter separatory funnel and after cooling to 37.5°C. is inoculated with a suspension of the organism to be studied. After thorough mixing to insure uniform distribution of the bacteria, aliquot portions (100 cc.) of the broth are run into flasks, the portions being accurately measured with a pipette connected to the funnel (fig. 1). Before use, the pipette is sterilized with the inlet and outlet tubes protected by plugged test tubes as shown for the outlet tube.

The test tube protecting the outlet should be replaced between each measurement of the aliquots.

The ordinary Florence flask with neck at least 10 cm. long (fig. 2) is recommended as a culture flask. Two sizes of glass tubing are selected. The smaller tube fits inside the larger (about 5 mm. inside diameter), leaving only a very small annular space. Over a length of about 4 inches near the end of the larger tube (15 cm. long) one layer of cotton twine is tightly wound. A washed rubber band may be used in place of the twine. This

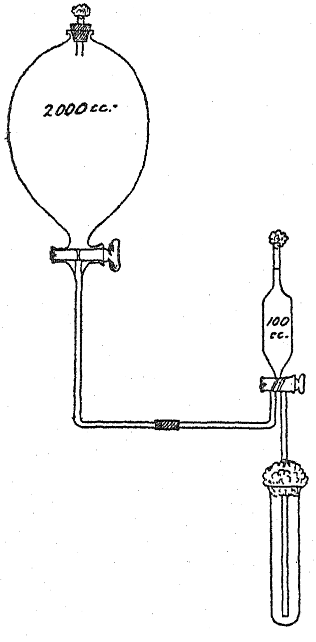

FIG. 1

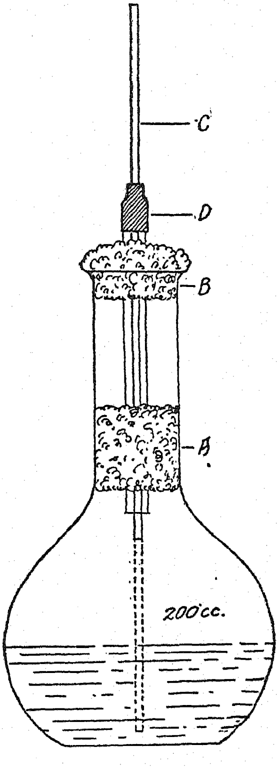

FIG. 2

FIG. 1. METHOD OF MEASURING ALIQUOTS OF INOCULATED BROTH
FIG. 2. CULTURE FLASK

serves to hold in place the plug of absorbent cotton which is wound in even layers (not too thick) over the twine or rubber, forming a neat compact plug, large enough to fit snugly into the lower end of the neck of the flask (*A*, fig. 2). A 4 mm. section of soft rubber tubing about 3 cm. long (*D*, fig. 2) is placed on the other end of this tube for a distance about half the length of the rubber tube. The smaller tube (22 cm. long) is then inserted in the larger, where it is held in place by the short section of rubber tube. A small plug of cotton is inserted in the upper end of the small tube. The plugged flasks are then sterilized. To minimize the possibility of contamination of the double plug device during the subsequent filling of the flask with inoculated broth, it is preferable to sterilize it in a test tube of approximately the same diameter as the neck of the flask, inserting it into the flask immediately after the filling.

The lower of the two plugs is moistened with 5 to 10 cc. of sterile 1 per cent zinc acetate solution as an absorbent for the hydrogen sulfide, the upper plug being withdrawn slightly to admit the slender tip of the pipette. The flask is then placed in the incubator. If desired, the smaller tube (*C*, fig. 2) may then be attached to a gas supply (hydrogen, carbon dioxide, air) with the lower end of the tube above the surface of the broth, or below the surface as shown by the dotted lines. For most purposes, one bubble per second is a satisfactory rate of flow for the gas through the medium. At the pressure thus created the zinc acetate solution in the lower plug should accumulate at its upper surface, providing a layer of solution about $\frac{1}{8}$ inch deep on top of the plug. If appreciable drying out of the lower plug occurs during the incubation period, more zinc acetate solution may be added without danger of contamination.

At the end of the period of incubation tube *C*, after being lifted out of the broth, is disconnected from the gas supply. The tube *C* is then withdrawn and 2 cc. of the broth is removed by inserting the elongated tip of the pipette through the larger tube. Bacterial counts are determined by the plate-dilution method. The rest of the broth and plugs *A* and *B*, after being unwound from the tube, are placed in the aerating cylinder and hydrogen sulfide is determined by the methylene blue method.

EXPERIMENTAL

Throughout the investigation 3 per cent pepton solution containing 0.5 per cent sodium chloride is employed as the culture medium. The initial hydrogen ion concentration is adjusted to approximately pH 8.0 before sterilization in every case, yielding a sterile solution of approximately pH 7.6. All cultures are incubated at 37°C.

TABLE 1

Hydrogen sulfide produced from peptones by Proteus vulgaris (100,000 per cubic centimeter) in twenty-four hours. Initial pH approximately 7.6

PEPTON	FINAL pH (APPROXIMATE)	FINAL COUNT OF BACTERIA PER CUBIC CENTIMETER	FINAL H_2S	RELATIVE H_2S FORMATION ($C = 1$)
			mmgm.	
A	7.4	225,000,000	738	24
B	7.6	370,000,000	584	19
C	7.4	240,000,000	31	1
D	7.4	260,000,000	215	7
E	7.4	300,000,000	473	15

Hydrogen sulfide from peptones

Tilley (1923) and others have shown that the quantities of hydrogen sulfide which may be yielded by peptones under the action of bacteria vary. The methods used by previous investigators for the determination of hydrogen sulfide formation were only approximately quantitative. Five brands of peptones, representative of those on the market today, were therefore tested by the new method.

For this experiment, the peptones were sterilized in the 200 cc. flasks in the autoclave for fifteen minutes at 15 pounds pressure. Ordinary plugs were inserted in the flasks for the sterilization, the double plugs being sterilized independently. Suspensions of *Proteus vulgaris,* made from vigorous twenty-four hour slant agar cultures, were so inoculated that the resulting broth contained 100,000 bacteria per cubic centimeter. The flasks were incubated for twenty-four hours with air bubbling throughout them at the rate of about one bubble a second. The results are shown in table 1.

All but one of the peptones gave a large volume of hydrogen sulfide. Peptones D, E, B and A yielded 7, 15, 19 and 24 times as much of the gas as pepton C. Another experiment, a duplicate of the first except that the cultures received an initial inoculation of 1,000,000 bacteria per cubic centimeter, gave practically identical figures for the amount of hydrogen sulfide produced in twenty-four hours, but the final bacterial count was greater in the second experiment than in the first. This indicates that the media were practically exhausted of the readily available material from which the gas was formed. The hydrogen ion concentration did not change appreciably during this short period.

Rate of hydrogen sulfide formation from pepton

It is well known that several hours are required for the growth of bacteria to become sufficiently vigorous to produce enough of the end products to give a positive test. The length of this "fore-period" would depend upon several factors, but under closely prescribed conditions it might be predicted to be solely a characteristic of the organism. Using a popular brand of pepton of American manufacture (pepton E), the rate of formation of hydrogen sulfide by *Salmonella aertrycke* and *Proteus vulgaris* was studied. Results typical of those obtained in several experiments are shown in tables 2 and 3.

The rate of hydrogen sulfide production is not uniform in the case of either of the bacteria. A sudden rise in hydrogen sulfide formation in the case of *S. aertrycke*, came some 30 minutes after an apparent rapid increase in microörganisms. At the period when the gas was first consistently detectable in the media, in the case of either culture, it was not possible to make observations fast enough to obtain the intermediate values which would show the initial slope of the rate curve. The maximum hydrogen sulfide production by *S. aertrycke* was about half that of *Proteus vulgaris*. *S. aertrycke* produced the maximum volume of gas in 6¼ hours. The other organism slowly produced hydrogen sulfide from the 13½ hour period to the 52-hour period, when the value was about 65 per cent higher than at the 13½-hour period.

From these data it may be tentatively concluded that when the bacteria have reached the stage of rapid multiplication they quickly attack the readily decomposable sulfur compounds present in the pepton, with the formation of hydrogen sulfide. Any further hydrogen sulfide formation depends upon the ability of the organism to split the residual complex. Apparently *Proteus vulgaris* possesses the power to decompose polypeptides in greater degree than *S. aertrycke.*

TABLE 2

Hydrogen sulfide produced from Pepton E by S. aertrycke. Culture not aerated

INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
hours		*mmgm.*
0	270,000	0
4½	109,000,000	0
4¾	14,000,000	8
5	1,400,000	0
5¼	31,000,000	Trace
5¾	117,000,000	0
6¼	190,000,000	202
7	267,000,000	202
8	158,000,000	196
9	245,000,000	196
9½	460,000,000	178
27	15,000,000	199
53	365,000	

TABLE 3

Hydrogen sulfide produced from Pepton E by Proteus vulgaris. Culture not aerated

INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
hours		*mmgm.*
0	1,000,000	0
8⅔		0
9½	190,000,000	Trace
13½	400,000,000	251
27½	600,000,000	267
31½	5,000,000,000	304
52	720,000,000	413

Influence of oxygen on hydrogen sulfide formation

Some interesting data were obtained on the effect of oxygen on *Proteus vulgaris* when grown in a solution of Pepton E (table 4).

Under the conditions of the test *Proteus vulgaris* grew better and produced hydrogen sulfide faster in the presence of oxygen than in its absence. The cultures which were not aerated followed a middle course between those aerated with hydrogen and those aerated with air. Similar results were obtained in other tests of like nature. Apparently decomposition of food products by this organism proceeds more quickly when the products are exposed to the air than when they are protected from it.

TABLE 4

Effect of air and hydrogen on hydrogen sulfide production from Pepton E by Proteus vulgaris

METHOD OF INCUBATION	TIME OF INCUBATION	BACTERIA PER CUBIC CENTIMETER	HYDROGEN SULFIDE
	hours		*mmgm.*
Not aerated	0	4,000,000	0
	5	24,000,000	181
	7	160,000,000	214
	27	150,000,000	434
	53	60,000,000	434
Aerated with air	0	4,000,000	0
	5	280,000,000	239
	7	500,000,000	355
	27	1,300,000,000	428
	53	3,100,000,000	420
Aerated with hydrogen	0	4,000,000	0
	5	130,000,000	217
	7	140,000,000	277
	27	20,000,000	336
	53	30,000,000	424

Hydrogen sulfide from cystin

Previous experiments had indicated that *S. aertrycke* and *Proteus vulgaris* quickly produced hydrogen sulfide from the readily decomposable sulfur compounds of the pepton. It was believed that preformed cystin comprises the greater part of the mother substances from which the gas is produced. If this is true the organisms should promptly decompose all of any cystin added to the culture medium and yield hydrogen sulfide from it in practically theoretical quantities. Accordingly *Proteus vulgaris* was allowed to grow in a solution of pepton E with and without the addition of the amino-acid cystin. Five cubic centimeters of sterile 0.2 per cent cystin dissolved in very weak hydrochloric acid solution (0.2 gram cystin + 20 cc. 0.075 N hydrochloric acid + water to 100 cc.) was added to 100 cc. of the sterile pepton solution in the usual 200 cc. flask. After inoculation, the medium was incubated at 37°C. and analyzed at intervals up to fifty hours total incubation period. The cultures, except in the case of one of the controls, were aerated at the rate of about two bubbles of air a second.

TABLE 5

Hydrogen sulfide produced from cystin by Proteus vulgaris

METHOD OF TREATMENT	INCUBATION PERIOD	BACTERIA PER CUBIC CENTIMETER	H_2S
	hours		*mmgm.*
0.01 gram cystin added; culture aerated	0	1,000,000	0
	2	14,000,000	0
	4	53,000,000	3.5
	5	360,000,000	6.8
	7	850,000,000	2,488
	25	1,030,000,000	3,310
	50	3,000,000,000	3,310
No cystin; culture not aerated	50	81,000,000	461
No cystin; culture aerated	50	3,200,000,000	461

The results (table 5) show that a slow bubbling of air is beneficial to the growth of *Proteus vulgaris*. The amount of hydrogen sulfide produced in fifty hours from broth without added cystin, whether aerated or not, however, was the same. In the growth with added cystin about 75 per cent of the total hydrogen sulfide obtained in fifty hours was produced between the fifth and seventh hours of incubation. Analysis showed that the cystin employed in the experiment contained 25.9 per cent sulfur (theory for chemically pure cystine, 26.67 per cent sulfur). Subtracting the weight of gas formed in the absence of cystin from that formed in its presence gives a value (2849 mmgm.) which agrees well with the quantity of hydrogen sulfide (2752 mmgm.) theoretically obtainable from the 0.01 gram of cystin. Considering the nature of the experiment, this agreement is very close and is believed to prove that all of the added cystin was decomposed.

TABLE 6

Hydrogen sulfide produced from pepton E by bacteria. Cultures aerated with air

BACTERIUM	HYDROGEN SULFIDE AFTER TWENTY-FOUR HOURS
	mmgm.
S. aertrycke	350
Esch. coli (variety *communior*)	15
Esch. coli (a)	462
Proteus vulgaris X19 (a)	60
Proteus vulgaris (a)	462
Proteus vulgaris (b)	458
Proteus vulgaris (c)	462

(a) Procured from Bacteriological Laboratory, University of Iowa, Iowa City, Iowa.

(b) Procured from Bacteriological Laboratory, Ohio State University, Columbus, Ohio.

(c) Bureau of Chemistry strain.

Hydrogen sulfide produced by different strains of bacteria

Numerous tests of the hydrogen sulfide producing power of different bacteria have been reported in the literature. In expressing the results the attempt was made to indicate an approximate quantitative relationship between the amounts of gas produced in a given time. Tilley (1923a) reports a variability in the amount of hydrogen sulfide produced by different bacteria in a beef infusion agar medium containing added cystin. He records the quantities in relative terms, as "small amount," "moderate amount," and "large amount." Similar variations were noted by Sasaki and Otsuka (1912), using Fränkel's solution, and by Myers (1920) and others. Apparently bacteria which can produce hydrogen sulfide in suitable media differ in the speed of their action, a difference probably related in part to the rate of growth. It was considered of interest to test various strains of *Proteus vulgaris* to determine whether the amount of hydrogen sulfide produced from pepton E, in the case of this organism, was a species characteristic or varied with the strain, and also to test the ability of two or three other organisms to produce the gas under the same conditions.

The organisms used are listed in table 6. The broths were inoculated with 1,000,000 bacteria per cubic centimeter, no counts being made on the cultures at the end of the twenty-four-hour period except in the case of *Proteus vulgaris* (c), which showed 2,000,000,000 per cubic centimeter. Three strains of *Proteus vulgaris* produced practically the same amount of hydrogen sulfide in twenty-four hours. The remaining strain, *Proteus vulgaris* X19, differing culturally from the other strains only in that it was somewhat erratic, produced about one-eighth as much. Two strains of *Esch. coli* differed widely in hydrogen sulfide formation, *S. aertrycke* gave lower results than the strains of *Proteus vulgaris* but higher results than when the culture was not aerated (table 2).

Production of mercaptans by bacteria

The odor of mercaptans may be detected in aqueous solutions when they are in concentrations as low as one part in a million (Watson, 1922), but as yet satisfactory quantitative methods for use in this range are not available. The most delicate qualitative test for mercaptans involves the use of isatin in concentrated sulfuric acid, but the reagent does not lend itself readily to the quantitative determination of this class of sulfur compounds.

The new method of analysis for hydrogen sulfide in bacterial cultures adapts itself admirably to the simultaneous determination of other reducing substances. Kimball, Kramer, and Reid (1921) have shown that mercaptans may be accurately estimated by titration with iodin. By placing on the end of the aeration train of the hydrogen sulfide apparatus a series of four additional bottles, the first being a gas trap, the second containing about 75 cc. of N/100 iodin, the third a measured quantity of N/100 thiosulfate solution appropriately diluted with water, and the fourth a weak starch solution, it is possible to measure the amounts of reducing substances, other than hydrogen sulfide, which are evolved from the medium. Mercaptans and thioethers, under these circumstances, would pass through the zinc acetate solution and be acted upon by the iodin. The mercaptans change to stable alkyl disulfides and the thioethers form iodin addition compounds which do not affect the iodin titre as the iodin is quantitatively liberated therefrom during the back titration with sodium thiosulfate solution. It is thus seen that the method is practically specific for mercaptans. The titration is carried out in a manner similar to that used by Fellers, Shostrom, and Clark (1924) for the determination of hydrogen sulfide. It is recognized that the suggested procedure has the disadvantage that it is limited in delicacy by the strength (N/100) of the thiosulfate solution required for the back titration. The efficacy of the method was tested by analyzing solutions containing known quantities of ethyl mercaptan. Duplicate determinations checked very closely, 98.3 per cent of the mercaptan being accounted for.

Repeated trials demonstrated that detectable quantities of mercaptans were not formed by *Proteus vulgaris*, *S. aertrycke*, or *Esch. coli* in a solution of pepton E when incubated for two days at 37°C. without aeration. It is believed, however, that interesting information on mercaptan formation by strict anaerobes could be obtained by this method in view of the observation by Rettger (1906) that mercaptans are formed in abundance by anaerobic organisms in pure culture.

SUMMARY

A method for following the rate of hydrogen sulfide production by bacteria in broth cultures recently devised in the Bureau of Chemistry may be successfully applied under proper conditions to the study of aerobes, facultative anaerobes, and strict anaerobes.

The ratios of the amounts of hydrogen sulfide produced by

Proteus vulgaris in twenty-four hours from five peptones on the market were found to be 1:7:15:19:24.

A large proportion of the total hydrogen sulfide yielded by *Proteus vulgaris* and *S. aertrycke* from pepton in two days is produced in a comparatively short time in the early part of the incubation period.

Proteus vulgaris grew better and produced hydrogen sulfide faster from pepton under aerobic conditions than in the absence of oxygen.

Cystin gave practically the theoretical amount of hydrogen sulfide in pepton inoculated with *Proteus vulgaris.*

Three out of four authentic strains of *Proteus vulgaris* gave the same amount of hydrogen sulfide from pepton in twenty-four hours.

Tests for the formation of mercaptans by *Proteus vulgaris, S. aertrycke,* or *Esch. coli* from pepton during a two-day incubation were negative.

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THE BUTTER AROMA BACTERIA

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It is not our present intention to consider the influence which the feeding of the cows or the various methods of ripening may have on the production of aroma in butter, and neither will we dwell on the various microörganisms, lactose fermenting yeasts and ester forming strains of *B. coli* which were supposed by former investigators to contribute towards aroma production but which we would now rather regard as harmful in butter. We will, however, at once pass on to discuss the remarkable capsule forming streptococci which were demonstrated almost simultaneously by Boekhaut (1917) in Holland, Storch (1919) in Denmark and Hammer (1919, 1920, 1921) in America, and to which all three investigators have ascribed an important influence in the formation of aroma in butter.

These bacteria form small amounts of acetic and carbonic acids and other volatile products, but hardly any appreciable quantities of lactic acid. According to Hammer, these substances would for the most part be formed at the expense of the citric acid in the milk, therefore he has called his bacteria *Sc. citrovorus* and *Sc. paracitrovorus*.

Practical trials with these aroma bacteria have, however, failed to give decisive results, so there is still a good deal to be said in favour of the current opinion that butter of good aroma can be produced only by the use of vigorous pure cultures of Storch's cream ripening bacterium which one of us has investigated thoroughly and named *Sc. cremoris* (Orla-Jensen, 1919). On this account it may well be surmised that the butter aroma bacteria are really only weakened forms of *Sc. cremoris*. Clarity on this point would appear to be the first condition necessary for the solution of the aroma question.

Nothing is, however, more difficult than to assign a place in the system to a bacterium without any really characteristic properties.

The microscope affords but little help. The aroma bacteria resemble other streptococci in appearance. They have been stated to be specially slender, but are by no means so in pure cultures; their thickness varies very considerably with the conditions of nutriment. It is true that the aroma bacteria as a rule form well marked capsules but this is also the case with all the other lactic acid bacteria in the incubation stage proper, before they have produced appreciable amounts of lactic acid, while, as mentioned above, the aroma bacteria form very little acid; *Sc. cremoris* forms far larger capsules when appearing as the ropy milk bacterium. This is an example of the general rule in Bacteriology that systematic classification can only be based on the biological properties of bacteria.

As we have hitherto never met with any streptococcus which has not turned out to be a lactic acid bacterium, we started from the assumption that the aroma bacteria must be lactic acid bacteria. The fact that the small amount of acid formed by the aroma bacteria is mostly acetic acid does not invalidate this assumption, for every weakened lactic acid bacterium growing under unfavourable circumstances, forms relatively large amounts of acetic acid.

Our only firm basis for departure is the ability of the aroma bacteria to ferment citric acid. We have been able to confirm the accuracy of this observation. Freshly isolated cultures are able to cause a vigorous evolution of carbon dioxide in milk with added citrates but as happens with the Betabacteria, they completely lose the power to produce gas after propagation for some time as pure cultures. This property is thus not a constant one and therefore possesses only a minor significance for the purposes of classification.

In reality the only citric acid fermenting bacteria known are certain aerogenes bacteria with which, however, the aroma bacteria have no connection whatever. We have tried whether any of the pure lactic acid bacteria could ferment citric acid, and have found that certain Betacocci possess this property to a fairly marked degree, and as they, like the aroma bacteria, generally have a comparatively low optimum temperature and form a number of by-products, there is some likelihood that aroma bacteria are connected with the Betacocci.

We give here a rough summary of the new classification of the lactic acid bacteria (Orla-Jensen, 1919) for the benefit of those who may not be acquainted therewith.

Sphere forms	*Rod forms*
a. Form only traces of by-products in addition to lactic acid	
Streptococci (always dextro lactic acid)	Streptobacteria (dextro or inactive lactic acid) Thermobacteria (laevo or inactive lactic acid)
b. A number of by-products in addition to lactic acid	
Betacocci (always laevo lactic acid (seldom inactive)	Betabacteria (inactive lactic acid)

It will be seen that the Betacocci are primarily distinguished by the formation of laevo lactic acid, and if therefore we are to prove that the aroma bacteria belong to this group, we must first try to find a nutrient medium which suits them sufficiently well to allow of their developing their activities to the full and thus revealing their hidden characteristics.

As milk does not seem to be a favourable medium for aroma bacteria in pure cultures, we naturally tried yeast extract which has proved to answer admirably for many of the rod shaped lactic acid bacteria.

The yeast extract used was prepared by the autolysis of pressed yeast, and adjusted to the same hydrogen ion concentration and the same content of nitrogen, phosphoric acid and milk sugar as in the milk used. As the yeast extract also had approximately the same high content of buffer substances as milk, there was hardly any other physiological difference between the two nutrient media than that in the composition of the nitrogenous matter; the effect produced by mixing them in certain proportions is thus mainly to be sought in modifications in the nitrogenous nutriment. We must, however, remark that we did not succeed in preparing absolutely sugar free yeast extract; this invariably

TABLE 1

KIND OF BACTERIA	NUMBER OF DAYS	$^{\circ}/_{00}$ LACTIC ACID									
		Pure yeast extract	Per cent of yeast extract in milk								Pure milk
			50%	35%	10%	5%	2.5%	1%	0.5%	0.1%	
Sc. citrovorus (Hammer)....	1	3.2	7.8	6.2	4.3	3.4	3.8	2.5	1.5	0.9	0.7
	7	4.1	9.4	8.1	5.5	4.4	4.6	2.5	1.5	1.7	1.4
Aroma bacterium (2x).......	1	0.7	3.4	2.7	1.8	1.8	1.7	1.4	1.0	0.8	0.5
	7	4.4	6.2	5.1	6.9	3.7	3.6	1.8	1.4	0.9	1.0
Aroma bacterium (6 Kirkedal 6)............	1	1.8	5.0	5.0	3.4	3.2	2.7	2.7	2.0	0.7	0.5
	7	3.4		7.7	10.6	6.5	6.0	3.6	2.9	1.6	0.9
Bc. arabinosaceus...........	1	1.4	5.3	4.7	3.1	1.5		1.5			1.2
	7	6.5	7.2	7.3	7.2	6.4		5.6			4.1
Bc. bovis..................	1	0.7	1.9	2.0	2.4	2.6		1.9			0.7
	7	4.5	5.0	6.9	6.5	5.8		5.4			4.1
Bbm. caucasicum............	1	5.4	7.4	6.7		2.5	1.9	1.7	1.5	1.3	0.8
	7	7.9	10.4	11.0	9.5	5.0	5.0	3.5	3.2	2.0	1.7
Bbm. breve.................	1	0.9	0	0	0	0	0	0	0	0	0
	7	15.6	17.3	11.7	6.5	5.0	4.7	4.2	3.7		0.6
Bbm. longum...............	1	6.7	3.1	1.8	0.7	0.7	0.4	0	0	0	0
	7	6.7	9.2	10.1	10.1	4.3	4.0	4.0	2.2	2.2	0.5
Sbm. casei.................	1	0.6	2.0	1.1	0.9	0.9	0.9	0.5	0.2		0.2
	7	14.1	14.6	16.1	15.9	14.9	13.7	13.1	13.1		11.7
Sbm. plantarum (1).........	1	0	0	0	0	0	0	0	0	0	0
	7	11.8	12.1	14.2	11.0	9.2	7.6	5.2	4.4		1.6
Tbm. lactis................	1	7.0	11.3	12.2	11.9	11.9	11.5	11.7	10.6	10.4	9.7
	7	14.1	17.6	18.7	19.7	19.4	18.6	17.8	17.8	17.8	17.1
Tbm. bulgaricum...........	1	4.5	10.8	12.2	12.2	12.2	13.3	12.2	11.9	12.2	11.5
	7	11.0	15.8	16.9	16.7	17.0	16.9	16.9	17.0	17.1	17.1
Tbm. Jugurt...............	1	2.3	11.0	14.9	16.2	16.0	14.9	14.9	14.9	14.9	14.0
	7	7.6	23.6	24.5	27.3	25.2	24.8	26.1	25.5	25.5	26.1

TABLE 1—*Concluded*

KIND OF BACTERIA	NUMBER OF DAYS	°/₀₀ LACTIC ACID									
		Pure yeast extract	Per cent of yeast extract in milk								Pure milk
			50 %	25 %	10 %	5 %	2.5 %	1 %	0.5 %	0.1 %	
Tbm. helveticum	1	3.6	10.1	13.7	11.7	11.5	11.3	11.5	9.9	9.9	9.7
	7	8.8	16.4	20.5	23.4	22.5	22.7	23.0	22.1	22.1	22.3
Sc. faecium	1	2.0	4.7	6.2	5.7	5.4	5.0	4.1	3.6		2.3
	7	2.0	5.0	6.2	6.3	6.0	5.6	5.5	5.5		5.1
Sc. glycerinaceus	1	2.3	4.6	5.8	5.3	4.5	4.5	3.6	2.6		1.5
	7	2.4	4.9	5.3	5.1	5.0	5.0	4.7	4.4		4.4
Sc. liquefaciens	1	1.9	4.6	5.9	5.4	5.4	5.9	5.6	5.6		5.4
	7	2.0	4.7	5.8	5.4	5.4	6.1	6.4	6.3		5.9
Sc. thermophilus	1	2.0	4.1	4.3	6.3	6.5	6.5	6.1	5.9	5.2	5.0
	7	3.6	6.4	7.7	9.1	9.1	9.1	8.8	8.3	8.1	7.9
Sc. mastitidis	1	3.0	3.0		5.1	4.9	4.4	2.3			0.9
	7	3.2	3.8		5.2	5.2	5.0	5.0			5.3
Sc. cremoris	1	2.0	5.0	6.3	7.2	7.2	6.8	7.2	6.8	7.0	6.5
	7	5.0	7.4	8.3	8.6	8.8	9.4	8.8	8.8	8.8	8.3
Sc. lactis	1	2.5	2.7	6.4	5.5	5.6	6.5	7.0	7.4		7.6
	7	2.5	2.9	6.1	5.6	5.4		7.1	7.4		7.4

contained a trace of glucose in addition to yeast gum. The addition to milk of these carbohydrates, even in appreciably larger proportions than those in which they occurred in the yeast extract, did not however, improve in any way the nutrient value of the milk towards aroma bacteria or true lactic acid bacteria.

We now tried not only the cultivation of aroma bacteria in pure yeast extract but also in various mixtures of milk and yeast extract. For the sake of comparison we further tried representatives of the most important lactic acid bacteria in the same way. In table 1 the amounts of acid formed (the acidity of the inoculated medium minus that of the not inoculated medium) are expressed as $^{\circ}/_{\circ\circ}$ of lactic acid.

As is seen from table 1, we have just hit on exactly the right mixture, and it thus becomes possible to solve the problem in question. With 10 to 50 per cent of yeast extract in milk, the aroma bacteria form just as much acid as other streptococci, so that we are in a position to analyse the acid produced. It turns out to be mostly lactic acid, and laevo lactic acid at that, so there is no doubt at all that the aroma bacteria belong to the Betacocci, in which case they cannot be degenerated forms of *Sc. cremoris*.

To determine whether the aroma bacteria ferment arabinose or not, i.e., whether they are more nearly related to *Bc. arabinosaceus* or to *Bc. bovis*, we have tried out their behaviour towards various sugars admixed with yeast extract and milk from which we had fermented away the milk sugar with the help of Kefir yeast; we also found it to be very nearly as satisfactory to employ the more simply prepared mixture of yeast extract and the solution of peptonised casein containing various salts which we generally use in this laboratory.[1] The results shown in table 2 were obtained with this solution containing 25 per cent of yeast extract.

TABLE 2

KIND OF BACTERIA	GLYCEROL	XYLOSE	ARABINOSE	RHAMNOSE	SORBITOL	MANNITOL	LAEVULOSE	GLUCOSE	MANNOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	RAFFINOSE	INULIN	DEXTRIN	STARCH	SALICIN
Sc. citrovorus	0.4	0.4	0.2	0.5	0.2	0.2	0.6	4.1	0.7	3.3	4.1	0.5	4.3	0.2	0.5	0.6	0.2	0
Aroma bacterium 2 x	0.2	0.4	0.5	0.4	0.2	0.2	0.6	5.0	0.4	2.4	0.7	0.6	4.7	0.2	0.4	0.5	0.4	0
Aroma bacterium 6 K 6	0.2	0.2	0	0.4	0.6	0.4	5.2	5.4	0.4	2.7	4.0	0.6	4.7	0.2	0.5	0.5	0.4	0.2
Aroma bacterium 1 x	0.4	0.4	0.2	0.4	0.1	0	0.4	4.6	0.6	3.2	4.1	0.2	0.5	0.2	0.2	0	0	0
Sc. paracitrovorus	0.4	1.0	1.0	0.2	0.1	0.2	3.6	3.7	2.7	2.4	5.8	4.2	3.6	3.2	0.2	1.1	0.1	0

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[1] Orla-Jensen: Dairy Bacteriology, p. 25. In different investigations we substituted for the extract of bakers' yeast generally used in this laboratory, one which was manufactured some years back from brewers' yeast (Cibus) and used for soup extracts; as a rule there was no difference in the amounts of acid produced.

It will be seen that the aroma bacteria display no marked propensity to ferment pentoses, for which reason they show closer affinity to *Bc. bovis* than to *Bc. arabinosaceus.*

The majority of the forms examined, however, differ, from *Bc. bovis* in having but little power to ferment laevulose, mannose and maltose, so possibly they constitute a distinct species. With the exception of 2 they ferment sucrose, and with the exception of 1 they ferment lactose. *Sc. paracitrovorus* has the power to ferment a larger selection of carbohydrates than the other aroma bacteria and also differs from them in growing very slowly at the ordinary temperature for which reason it can hardly have any practical significance.

Referring back to table 1, we see, as might be expected, that the development of the Betacocci is also greatly stimulated by the addition of yeast extract. This effect is even more pronounced in the case of the nearly related Betabacteria; as one of us has already mentioned,[2] *Betabacterium caucasium* which constitutes the major portion of the Kefir grain, hardly grows at all in milk, and only forms appreciable amounts of lactic acid in the Kefir grains, i.e., in symbiosis with the yeast cells present therein which of course corresponds with the addition of yeast extract.

The rod shaped lactic acid bacteria called the Streptobacteria, among which *Sbm. casei* plays an important part in the ripening of cheese, also thrive much better in yeast extract than in milk, and best in milk containing about 25 per cent of yeast extract.

Even in the case of the lactic acid bacteria which grow better in milk than in yeast extract, the addition of a certain proportion of yeast extract has a favourable influence both on the rapidity of growth (amount of acid after twenty-four hours) and on the formation of acid (amount of acid after one week), but naturally the optimum proportion of yeast extract is somewhat lower for these than for those bacteria which prefer pure yeast extract to pure milk. Only *Sc. lactis* (the ordinary bacterium of sour milk) does not appear to improve on the addition of yeast extract.

It is particularly interesting to notice the stimulating effect of even quite small amounts of yeast extract on the development of lactic acid bacteria. Even *Sc. cremoris* which prefers milk to yeast extract is noticeably stimulated both in growth and power to form acid by the addition of as little as 1 per cent of yeast extract.

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[2] Orla-Jensen, 1919, p. 177.

If we are dealing with degenerated forms, the effect is much more marked. As regards the aroma bacteria we have even found cases of freshly isolated strains being able to coagulate milk on the addition of 1 per cent of yeast extract. We may further mention that a mixed culture of aroma bacterium and of a *Sc. cremoris* which ceased to grow at 6°C. in pure milk, formed notable amount of acid at 4°C., and even appreciable amounts at 2°C. (though only after a month's time) after the addition of 1 per cent of yeast extract.

As the yeast extract only contains 7 per cent of solids, and furthermore only a very small proportion of this can be assumed to be active matter, we must here be dealing with the effects of very minute amounts, which affords a striking parallel to the case of the vitamines. In this connection we may refer to Barthel's recent demonstration (1924) that extracts of moulds will also stimulate the development of lactic acid bacteria.

Now as the lactic acid bacteria and not least the butter aroma bacteria, are just as particular as to their nutritive requirements as the higher animals, while on the other hand, yeast extract is rich in vitamines B and D, it is highly probable that we are dealing with the effects of these very substances, and we reserve the right to investigate this matter further in the hope of finding an easy manner of estimating one of these vitamines.

In order to isolate the butter aroma bacteria Storch and others have proposed certain cumulative methods. These are, however, not applicable if the object is to form an opinion as to the proportion of aroma bacteria to other bacteria in a starter culture. To assume that aroma bacteria can be recognized by direct microscopic examination will lead to very serious mistakes. It might be thought that aroma bacteria would be easy to discover in litmus gelatin, but neither does this method lead to satisfactory results as on the one hand the aroma bacteria are quite capable of turning litmus red when fresh from the starter culture, while on the other hand, cells of *Sc. cremoris* are often found in the starter culture which are very weak acid formers. In view of the above results we hoped a great deal from a casein pepton gelatin with yeast extract and cane sugar, as these substances promote the growth of aroma bacteria to a greater extent than that of the other bacteria of the starter. This medium, however, did not turn out to be very selective as most other lactic acid bacteria grew well on it simultaneously.

In order to judge of the number of aroma bacteria in proportion to other lactic acid bacteria, we have no better method than to plate the starter out on our ordinary glucose, casein, pepton gelatin, to isolate a large number of strains at random and to examine these in detail.

As these investigations will be further treated of in a separate paper we will not dwell on them here but only point out that the aroma bacteria seem as a rule to occur very scantily indeed in proportion to *Sc. cremoris.* From most starter cultures we did not succeed in isolating any aroma bacteria at all without previous enrichment. The fact that they were always present, however, was shown by the circumstance that when we inoculated the culture into milk with chalk, appreciable amounts of inactive (laevo + dextro) lactic acid were always formed besides the dextro acid.

The fact that the development of the aroma bacteria as well as the formation of acid can be furthered by the addition of a little yeast extract, seems to open up new possibilities in relation to the problem of cream ripening. One cannot of course add yeast extract to the cream, this would be too expensive and also cause a taint, but there would be no obstacle in the way of adding a little yeast extract to the milk used for the cultures or starters.

Unfortunately our laboratory experiments have not given any encouraging result in this direction. The vitality of the lactic acid bacteria does not seem to be enhanced by the use of yeast extract, but the bacteria would rather seem to become pampered so that when afterwards cultivated in pure milk (or pure cream) they form a little less acid than previously to the cultivation with yeast extract.

Nevertheless we are of the opinion that by further investigations of the ripening problem, the addition of yeast extract as well as of citrates are matters which should also be tested out.

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PHYSIOLOGICAL STUDIES OF ASSOCIATION

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INTRODUCTION

This paper is the second of a series of articles dealing with certain factors which influence the rate of cellulose fermentation (see Sanborn, 1926). In the present work the influence of microbial association is investigated.

One of the most prominent organisms employed in association experiments is *Azotobacter*, significant because of its ability to synthesize the growth-accessory factor, and, with regard to its present application, because of the intimate connection between nitrogen assimilation by *Azotobacter* and cellulose decomposition. Hunter (1923) has shown that *Azotobacter* is capable of synthesizing an accessory factor similar to vitamine B. Mockeridge (1924) noted the growth-promoting effect of *Azotobacter* and *Rhizobium* upon green plants.

The work of Hutchinson and Clayton (1919) indicates that products of cellulose decomposition stimulate nitrogen assimilation by *Azotobacter*. Similar results have been obtained by Pringsheim (1909, 1910).

A cellulose-destroyer, *C. folia*, isolated by the author from decomposing leaves, proved to be a suitable organism upon which to determine the influence of association. The organisms selected for association with *C. folia* were forms which the author has found to be invariably connected with active cellulose destruction in nature, and were isolated from mixed cultures in which cellulose was undergoing rapid decomposition. These organisms are as follows: *Act. colorata*,[1] *Azotobacter*, *B. mycoides*,

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[1] *Act. colorata* is a new species, described elsewhere by the author.

B. subtilis, and *B. cereus*. For details in technique and method, reference may be made to the first paper of the series.

EXPERIMENTAL

a. The influence of association with Act. colorata upon the growth and physiological efficiency of C. folia

Investigations by the author showed that hydrogen-ion concentration determinations would serve as a legitimate criterion of the rate of cellulose decomposition by *C. folia* and as a measure of its physiological efficiency. A basic nutrient medium was employed which lent itself readily to the H-ion technique.[2] This medium proved suitable mainly because its "buffer index" is negligible and because it will not support the growth of *C. folia*. For the fermentation experiments raw cotton was added to the nutrient medium. The electrometric method described by Itano (1923) was used throughout the investigation, supplemented by the colorimetric method.

The same general technique was employed as in the experiments previously reported. For the H-ion series, liter Erlenmeyer flasks, containing 200 cc. of the nutrient medium and 3 grams of raw cotton, received inoculations from a twenty-four hour nutrient solution culture of *C. folia*, and from a four-day old culture of the *Actinomyces* in nutrient solution containing raw cotton. Incubation took place at 27°. The results (tables 1 and 2) show the influence of *Act. colorata* upon the growth and physiological efficiency of *C. folia*. Because of the filamentous character of the *Actinomyces* growth and its tendency to break up into segments and form conidia, an accurate plate count, particularly with the *Actinomyces* above, proved impossible.

Tables 1 and 2 indicate that *Act. colorata* exerts a stimulative action upon the growth and physiological efficiency of *C. folia* *at the expense of the former organism which gradually dies out.* In the fermentation flasks, in five weeks' time no trace of the *Actinomyces* could be found. In order to determine more definitely the nature of the stimulation, an extract was made from the *Actinomyces* cells. Cells from a four-day-old culture growing upon N-free agar were washed in saline solution and then suspended in nutrient medium. The suspension was acidified to pH 4.6 and boiled for ten minutes on each of two consecutive days.[3] The extract contains the water-soluble substances pres-

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[2] The medium is a modification of McBeth's solution, and is described in the first paper of the series.

TABLE 1

The influence of Act. colorata upon the growth of C. folia

DAYS	CONTROL		ASSOCIATION OF C. FOLIA AND ACT. COLORATA IN BASIC NUTRIENT SOLUTION WITH CELLULOSE	
	Act. colorata alone	*C. folia* alone	*C. folia*	*Act. colorata*
1	100*	30†	55†	100*
2	Rapid increase in growth	42	98	66
3	Filaments form segments	54	137	46
4		66	148	30
5	Conidia appear	78	146	22
6	1,000	100	130	20
7	1,000	150	110	18

* Expressed in thousands.

† Expressed in millions.

TABLE 2

The influence of the association of cellulose-destroyers upon the rate of cellulose decomposition

DAYS	C. FOLIA ALONE	ACT. COLORATA ALONE	ASSOCIATION OF C. FOLIA AND ACT. COLORATA
	pH	*pH*	*pH*
Initial	8.40	8.40	8.40
4	7.84	7.56	7.30
8	7.30	7.18	6.20
12	6.96	7.00	5.34
16	6.76	6.90	5.00
20	6.68	6.84	4.84
24	6.66	6.78	4.80
28	6.64	6.70	4.80
42	6.46	6.30	
56	6.16	6.00	
63	6.00	5.82	
70	5.80	5.60	

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[3] The work of Wildiers and others show "Bios" to be unaffected by boiling in acid environment. Method from Thjötta and Avery (1921).

ent in the living *Actinomyces* cells, and inasmuch as the failure of the *Actinomyces* results in the activation of *C. folia*, such an extract should contain the same essential food substances and produce stimulation. The results are given in table 3.

The results of the above experiments show that association with *Actinomyces* causes a stimulation in the growth and cellulose-decomposing ability of *C. folia*. The association may be considered equivalent to the addition of some essential food factor.

TABLE 3

The influence of extracts from cells of Act. colorata upon the growth of C. folia in basic nutrient solution

Expressed in millions

HOURS	EXTRACT FROM ACTINOMYCES CELLS	CONTROL (NO EXTRACT)
Initial	2	2
5	9	3
10	16	3
15	23	4
20	30	5
25	38	6
30	56	7
35	79	8
40	101	9
45	116	9
50	125	10
55	133	11
60	140	10
65	148	8

b. The influence of Azotobacter upon the growth and physiological efficiency of C. folia

One loopful from a five-day-old culture of *Azotobacter* (A4 Jones) on Ashby agar was introduced into 100 cc. of the basic nutrient solution. A uniform suspension was made. A suspension of *C. folia* from a twenty-four-hour nutrient agar culture was also made in 100 cc. of the nutrient solution. One-cubic centimeter portions from this suspension, containing approximately 3,000,000 cells, were used to inoculate test tubes, each containing 8 cc. of nutrient solution.

From the *Azotobacter* suspension dilutions were prepared in nutrient solution. One-cubic centimeter portions from these dilutions were also added to the tubes of nutrient solution. Two hundred *Azotobacter* cells were added in one case; 40,000 in a second; and in the last set, 2,800,000 cells. The living *Azotobacter* cells added were determined by the plate method using Ashby agar. Table 4 gives the results. These figures show that association with *Azotobacter* results in a marked stimulative effect upon the growth of *C. folia.* In order to ascertain more definitely the seat of the factor of stimulation, the influence of *Azotobacter* was investigated more in detail. The following preparations were made from a five-day-old culture of *Azotobacter* cultivated in Ashby solution without $CaCO_3$.

TABLE 4

The influence of Azotobacter upon the growth of C. folia in nutrient solution

HOURS	AZOTOBACTER, 200 CELLS, + C. FOLIA	AZOTOBACTER, 40,000 CELLS, + C. FOLIA	AZOTOBACTER, 2,800,000 CELLS, + C. FOLIA	CONTROL C. FOLIA ALONE
Initial	3,000,000	3,000,000	3,000,000	3,000,000
6	1,950,000	2,950,000	3,800,000	1,150,000
12	1,300,000	2,850,000	4,350,000	650,000
18	900,000	2,650,000	4,700,000	300,000
24	550,000	2,400,000	4,850,000	100,000
30	300,000	2,000,000	4,450,000	10,000
36	150,000	1,550,000	3,500,000	10,000
42	60,000	1,200,000	1,600,000	10,000−
48	10,000	1,000,000	1,000,000	10,000−

1. Suspension of washed *Azotobacter* cells (living)
2. Extract from *Azotobacter* cells
3. Extract from culture medium

The culture of *Azotobacter* was centrifuged, throwing down the cells and leaving the supernatant liquid clear. The latter was retained for the preparation of the extract from the culture medium. The cells were washed several times with sterile physiological salt solution, after which a cell suspension in 10 cc. of saline solution was prepared.

A similar suspension was used in preparing the extract from the

Azotobacter cells. In this case the saline suspension of washed cells was acidified to pH 4.6 as in the previous experiment, and boiled for ten minutes. The suspension was boiled only once.

From the supernatant liquid obtained after centrifuging the original culture, the extract from the culture medium was made. This liquid was filtered through a sterile porcelain filter and the resulting extract remained sterile. The influence of these preparations upon the growth of *C. folia* was determined as before.

Table 5 indicates that the stimulative effect of *Azotobacter* upon the growth of *C. folia* is caused by association with the living *Azotobacter* cell, and not with extracts from the cell or culture medium.

TABLE 5

The influence of washed Azotobacter cells; of extract prepared from Azotobacter cells; and of extract from the culture medium in which Azotobacter has been growing, upon the growth of C. folia

HOURS	WASHED AZOTOBACTER CELLS	EXTRACT FROM AZOTOBACTER CELLS	EXTRACT FROM CULTURE MEDIUM
6	750,000	100,000	20,000
12	1,020,000	20,000	10,000
18	1,310,000	10,000	5,000
24	1,560,000	10,000	5,000
30	1,670,000	10,000	5,000−
36	1,700,000	10,000	5,000−
42	1,650,000	10,000	5,000−
48	1,460,000	5,000	5,000−

To determine the influence of *Azotobacter* upon the physiological efficiency of *C. folia*, a twenty-four-hour nutrient solution culture of *C. folia* was made, also a suspension of *Azotobacter* in nutrient solution. Approximately equal numbers of each species were used as inocula (500,000,000 cells). The fermentations were carried on as in the previous experiment using 3 grams of raw cotton in 200 cc. of nutrient solution and incubating at 27° (see table 6).

This experiment shows that *Azotobacter*, itself not a cellulose-destroyer, exerts a stimulating action upon *C. folia* both in growth and in cellulose-decomposing ability. This influence is manifest when *C. folia* is in association with living *Azotobacter* cells.

The work was repeated and a similar result obtained using *Azotobacter chroococcum* which was isolated from soil in which active cellulose decomposition was taking place.

c. The influence of B. mycoides, B. subtilis, and B. cereus, upon the growth and physiological efficiency of C. folia

Following the same general technique, *B. mycoides*, *B. subtilis*, and *B. cereus* were investigated for their stimulating action. Preliminary experiments based upon visual tests indicated that these organisms, associated with active cellulose fermentation in soil, exerted an accelerating influence upon the rate of cellulose decomposition. Because of the inability of these organisms to multiply in the basic nutrient medium or to attack cellulose, their numbers rapidly decreased. This decrease could not be traced quantitatively by the plate method because of the similar nutritive proclivities of these organisms and *C. folia*. Microscopic examinations, however, revealed the gradual dying out of the associated bacteria, resulting at the same time in a marked stimulation of *C. folia* in growth and physiological activity. These organisms were cultivated upon nutrient agar (pH 7.60). Aqueous suspensions of the organisms were made, and after washing and centrifuging the cells, extracts were prepared as before by acidifying the washed cell suspension to pH 4.60 and

TABLE 6

The influence of Azotobacter upon the physiological efficiency of C. folia

DAYS	C. FOLIA ALONE	AZOTOBACTER ALONE	CONTROL: CHANGE IN pH OF MEDIUM WITHOUT ORGANISMS	ASSOCIATION OF C. FOLIA AND AZOTOBACTER
	pH	*pH*	*pH*	*pH*
Initial	8.40	8.40	8.40	8.40
2	7.90	8.30	8.34	7.24
4	7.40	8.24	8.28	6.52
6	6.98	8.16	8.22	6.08
8	6.68	8.10	8.16	5.80
10	6.48	8.04	8.12	5.58
12	6.42	7.96	8.06	5.46
14	6.40	7.90	8.00	5.40

boiling for ten minutes. In this particular experiment the boiling process was repeated upon three consecutive days. All of the extracts were sterile. The influence of these extracts upon the growth of *C. folia* was tested in tubes of nutrient solution, treated uniformly as in the previous experiments. *C. folia* was plated out at intervals upon nutrient agar (see table 7).

TABLE 7

The influence of extracts prepared from the cells of B. mycoides, B. cereus and B. subtilis, upon the growth of C. folia in nutrient solution

Expressed in millions

HOURS	CONTROL (NO EXTRACT)	EXTRACT FROM B. MYCOIDES CELLS	EXTRACT FROM B. CEREUS CELLS	EXTRACT FROM B. SUBTILIS CELLS
Initial	2	2	2	2
5	3	19	13	11
10	3	23	18	22
15	4	28	23	33
20	5	33	28	44
25	6	38	34	56
30	7	49	39	67
35	8	63	45	78
40	9	77	50	89
45	9	84	54	105
50	10	85	56	124
55	11	87	59	142
60	10	88	61	161
65	8	89	64	175

TABLE 8

The influence of B. mycoides, B. subtilis, and B. cereus upon the physiological efficiency of C. folia

DAYS	CONTROL, C. FOLIA ALONE	ASSOCIATION OF B. MYCOIDES WITH C. FOLIA	ASSOCIATION OF B. SUBTILIS WITH C. FOLIA	ASSOCIATION OF B. CEREUS WITH C. FOLIA
Initial	8.40	8.40	8.40	8.40
2	7.73	6.54	6.92	7.10
4	7.24	6.37	6.16	6.66
6	6.96	6.28	6.07	6.48
8	6.80	6.20	5.98	6.42
10	6.77	6.14	5.91	6.32
12	6.76	6.07	5.83	6.24
14	6.74	6.00	5.75	6.15

These results show that some essential food factor may be extracted from the cells of these organisms which accelerates the growth of *C. folia* in nutrient solution. To determine the influence of this factor upon the physiological efficiency of the cellulose-destroyer, the fermentation flasks, containing nutrient solution and raw cotton, received inoculations of 300,000,000 cells of *C. folia* and 50,000,000 cells of the associated organism. Table 8 gives the results of the association.

B. mycoides, *B. subtilis*, and *B. cereus* exert a stimulating action upon the growth and physiological efficiency of *C. folia.*

d. The study of microbial associations by means of the China blue-rosolic acid-cellulose medium

In association studies such as those carried on in this investigation, there is often need of a medium which will reveal in detail the results of association. In this experiment such a medium is described. Through its use the influence of association in cellulose fermentation may be observed conveniently and with a fair amount of accuracy. The method is based upon the China blue-rosolic acid reaction described by Bronfenbrenner (1918). The preparation of this "CR-cellulose" medium is as follows:

Basic nutrient solution[4]	1000 cc.
Raw cotton (chemically untreated)	30 grams

The salts are dissolved in 500 cc. of distilled water, and of the remaining 500 cc. a 0.5 per cent agar is prepared. Before sterilization the two portions are mixed and the cotton added, cut into small fragments. With constant stirring, 1 per cent "CR" preparation[5] is introduced. In transferring the medium to petri plates, care is taken to insure a uniform distribution of cotton over the plate. The dishes are then autoclaved. The percentage of agar present proves very satisfactory in offering a smooth homogeneous surface, yet allowing the microbial suspensions added to reach the entire mass of cotton. The pH of the medium prepared in this way is 8.40 and is colored a brilliant red.

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[4] Referred to previously. Described in first paper of series.

[5] "CR" indicator is obtained by mixing equal parts of 0.5 per cent aqueous solution of China blue with 1 per cent solution of rosolic acid in 95 per cent alcohol. On the alkaline side China blue is water clear. Rosolic acid gives on the acid side different shades of pale yellow, which is masked by the deep blue of the China blue and gives sharp color values in media. In alkaline environment, China blue being colorless, the rosolic acid gives a pure red.

After cooling, the surface of the medium is inoculated uniformly with the cellulose-destroyer; one-half of the plate only receives treatment with the associated organism. The indicator has its turning point very close to neutrality, and with the production of acid the medium changes to a deep blue.

TABLE 9

The influence of association upon the rate of cellulose fermentation as determined by the "CR-cellulose" reaction

DAYS	C. FOLIA[*] ALONE	C. FOLIA IN ASSOCIATION WITH:				
		B. mycoides	*B. cereus*	*B. subtilis*	*Act. colorata*	*Azotobacter*
Initial	Medium red (8.40)	Red	Red	Red	Red	Red
1	Medium red	Red	Red	Red	Red	Red
2	Medium red	Red	Red	Red	Red	Red
		(Slight reduction of indicator observed)				
3	Medium red	Blue ++[†]	Blue +	Blue ++	Blue ++	Blue +++
4	Medium red	Blue +++	Blue +	Blue ++	Blue ++	Blue +++
5	Medium red	Blue +++	Blue ++	Blue +++	Blue +++	Blue +++
6	Medium red	Blue +++	Blue +++	Blue +++	Blue +++	Blue +++

[*] *C. folia* alone caused the acid reaction in eight to ten days.

[†] + = medium slightly bluish (pH 6.8); ++ = blue color definite (pH 6.4–6.6); +++ = intense blue (pH 6.0–6.2).

All of the organisms investigated above were tested out upon this medium in association with *C. folia*. Table 9 shows the influence of association upon the rate of cellulose decomposition as revealed by H-ion concentration changes, using the "CR-cellulose" medium.

The results observed with this medium are quite in accord with the quantitative results recorded above. The greatest stimulation seems to occur when *C. folia* is associated with *Azotobacter*, *B. subtilis*, *B. mycoides*, or *Act. colorata*. In the case of *B. cereus* the initial stimulation is not as marked; in the course of twenty-five days, however, the acceleration in physiological efficiency caused by association with *B. cereus* even exceeds that caused by association with the other soil bacteria.

The value of the China blue-rosolic acid-cellulose medium lies in the ease of preparation, its adaptability to the H-ion technique, and the accuracy with which the indicator responds to stimulation in the physiological efficiency of cellulose-destroyers. Other advantages of the medium are as follows: The buffer index is negligible; the color change occurs with definiteness at very nearly pH 7.0; the indicator is not affected by heat, shows no evidence of bactericidal action, is comparatively stable; and its constituents are definitely known chemical substances (Bronfenbrenner, 1918).

TENTATIVE CONCLUSIONS

1. Association with *Act. colorata*, *Azotobacter*, *B. subtilis*, *B. mycoides*, and *B. cereus* causes a stimulation in the growth and physiological efficiency of *C. folia.*
2. Stimulation by *Act. colorata* takes place at its own expense. Some essential food substance is apparently furnished by the dead cells.
3. In association with *Azotobacter* the stimulation is brought about by the living cell.
4. *B. subtilis*, *B. mycoides*, and *B. cereus* secrete substances within the cell which function as an essential food factor for *C. folia.*

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THE RELATION BETWEEN THE COMPLEMENT FIXATION AND THE SKIN REACTIVE ANTIGENS OF THE TUBERCLE BACILLUS

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The studies which have been going on in this Laboratory for the last three years upon the antigenic relations of the tubercle bacillus have all tended to show that the substances which are responsible for allergy in the tuberculous animal are not identical with those which react with specific serum antibodies. The original studies by which this work was initiated yielded distinct indications that the mechanism of allergy was not identical with that underlying protein anaphylaxis. Subsequent investigation of the tubercle bacillus products (Zinsser, 1921; Zinsser and Parker, 1923) led to the recognition of a substance spoken of in this Laboratory as "residue antigen," easily obtained both from tubercle bacillus extracts and from broth cultures. This substance reacted with antibodies in precipitation tests and gave satisfactory complement fixation reactions, provided that appropriate quantitative proportions were maintained. This "residue" substance—which, incidentally, was obtained from many different bacteria—is analogous to the soluble specific substance described and analyzed by Heidelberger and Avery (1923), and, according to the studies of Mueller and his associates is probably of a carbohydrate structure.

Subsequently, Mueller (1926), starting his preparation from filtrates of tubercle bacillus cultures grown on a synthetic medium, succeeded in separating the substance which gives the specific precipitation reaction in vitro from that which induces skin reactions in tuberculous animals. He described the former substance, namely the "residue," as a non-protein gum, the skin reactive material as "probably but not surely a protein." At that time he also reported that the non-protein gum or "residue" gave complement fixation reactions. His results corresponded with considerable accuracy to those of Laidlaw and Dudley (1925) published just before the appearance of his own paper.

The work herein reported was largely undertaken because of publications by Dienes and Schoenheit (1925) and Dienes, Schoenheit and Scheff (1925). These writers, who have had considerable experience in the chemistry and immunology of tuberculosis, obtained results which indicated to them that there was a close parallelism between antigenic preparations which fixed complement and those which gave positive skin tests in tuberculous animals. In their general conclusions they imply an identity of the two factors and the probability that the active substance is of a protein nature.

Our own experiments were undertaken in order to check up the previous results outlined above and to make sure that there had been no error in the work from this Laboratory which seemed, in the respects mentioned, diametrically in contrast to the work of Dienes and his collaborators.

The antigens used in the experiments were prepared as follows:

I. Twenty cubic centimeters of O.T. was mixed with 20 cc. of salt solution and centrifuged until clear. The supernatant fluid was precipitated with two volumes, namely, 80 cc. of alcohol, the sediment of this precipitation to be referred to as N I. To the supernatant fluid was added 120 cc. of alcohol, this making five volumes, and the precipitate formed again removed by centrifugation. The sediment of this precipitation, which was marked N II, was set aside and the supernatant fluid evaporated in the water bath down to 40 cc. This evaporate was marked N III. The sediments N I and N II were then taken up respectively in 40 cc. of saline solution. There were then three fractions obtained by successive alcohol precipitation and the evaporation of the remainder to the original volume. Complement fixations and skin tests were then carried out with these three materials as antigens against the same antituberculosis serum as follows:

	ANTIGEN DILUTION					
	1:10	1:100	1:1000	1:10,000	1:50,000	1:100,000
N I	++++	++++	+	−	−	−
N II	++++	++++	++++	±	−	−
N III	+++	±	−	−	−	−
O.T.	++++	++++	++++	++++	+	−

Skin test

N I: 10	−
N II: 10	++
N III: 10	++++
O.T.: 50	++++

This experiment confirms the work previously published by Mueller and is in keeping with the complement fixation work on tuberculosis residue of Zinsser and Parker, in that it shows definite complement fixation by the residue material which is, though crudely made in this case, relatively protein-free. In harmony with Mueller's experiments, moreover, this protocol indicates that the skin reactive factors of the series of fractional parts are directly the reverse of the complement fixation properties and in no respect therefore comparable to the results reported by Dienes and his co-workers.

Experiments like the one above were repeated with a number of other fresh preparations with like results.

Since fractional separations of tubercle bacillus antigens were made not only from O.T. in the previous work carried out in this Laboratory, but also from extracts of the tubercle bacilli themselves, we carried out similar experiments by fractional alcohol precipitation of watery extracts of tubercle bacilli which had been treated with ether and alcohol.

II. After fat extraction, the bacilli were ground with water in a ball mill for 14 hours. The extract was then filtered and centrifuged. Fractional alcohol precipitation was carried out exactly as before. N I, N II and N III in this experiment signify fractions analogous to those similarly named in the preceding experiment.

	ANTIGEN DILUTION			
	1:10	1:100	1:1000	1:10,000
N I	++++	+++	++	−
N II	++++	++++	+++	−
N III	+	−	−	−
Watery extract		++++	++++	++

Skin test

N I:10	±
N II:10	+
N III:10	++++
Watery extract	++++

This experiment may be interpreted exactly as the one above.

CONCLUSIONS

We have above reported experiments in which successive fractions of O.T. and of watery extracts of tubercle bacilli were tested in regard to their relative values as antigen for complement fixation and as materials for the eliciting of skin reactions in tuberculous guinea pigs. Our results have unequivocally shown that when tubercle bacillus preparations are treated with alcohol in the manner indicated the fractions which show the highest complement fixation potency are least active for skin reaction, and vice versa. We think it likely that the two functions do not reside in the same chemical constituent of the tubercle bacillus or of old tuberculin.

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A CAUSATIVE FACTOR OF "FLOATERS" DURING THE CURING OF OLIVES

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The occurrence of blister covered olives, commonly called "floaters," and the rapidity with which whole vats may become infected during processing, has made the identification of the causative agent and the control of this form of spoilage a problem of considerable importance. The olives are covered with blister-like protuberances which, if pricked with a needle, give off a small amount of gas followed by brine; the wrinkled skin subsequently fills a cavity in the flesh. On account of the gas content the fruit floats on the brine; the designation "floater" is, therefore, quite appropriate. On section, the olives are riddled with gas pockets around which the flesh is quite soft. The report of Cruess and Guthier (1923) very aptly describes and illustrates this condition. It is generally known that the large olives—Manzanillo and Sevillano varieties—are particularly suceptible to this type of spoilage. In order to determine whether or not the causative factor was bacterial or chemical, the following series of experiments were carried on with three lots of ripe California olives.

GENERAL PROCEDURE

Large ripe olives, which during processing had become "floaters" were brought to the laboratory directly from the infected vats. These were characterized by blisters covering part or all of their surface, and their consequent ability, because of this gas, to float on the surface of their liquid environment.

The individual olives were immersed for a few moments in 1:1000 bichloride of mercury solution and then transferred to several washings of saline. The blisters were cut with a sterile scalpel and bits of the soft flesh inside of the pockets were transferred to glucose broth. The cultures were incubated in duplicate sets, part at 37°C. and part at 28°C. to determine the optimum temperature for growth. Direct smear from the olive tissue showed a few rather large, irregular staining rods, apparently Gram-negative.

After twenty-four hours incubation, there was growth and copious gas production in the tubes incubated at 37°C., but less turbidity and a smaller amount of gas in those held at 28°C. Streaks were made from this glucose broth onto glucose agar plates, which yielded almost pure cultures of a Gram-negative rod, the only other oganism present being a small Gram-positive coccus. No anaerobes were demonstrated.

TABLE 1

ORGANISM	GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MALTOSE	DEXTRIN	XYLOSE	INULIN	GALACTOSE	LEVULOSE	DULCITOL	ARABINOSE	RAFFINOSE	SORBITOL	SALICIN
H.........	⊕	⊕	⊕	⊕	⊕	+*	⊕	†	⊕	⊕	†	⊕	⊕	⊕	⊕
M.........	⊕	⊕	⊕	⊕	⊕	+*	⊕	†	⊕	⊕	†	⊕	⊕	⊕	⊕
F..........	⊕	⊕	⊕	⊕	⊕	⊕	⊕	†	⊕	⊕	⊕	⊕	⊕	⊕	⊕

+ = acid.
⊕ = acid and gas.
* Trace.
† Growth.

CULTURAL CHARACTERISTICS

When repeated subculturing substantiated the fact that the culture which for the sake of brevity is designated as "H," was pure it was subjected to the routine biochemical tests. The reactions in the various sugars are shown in table 1. (The organisms "M" and "F" included in this table will be considered further on.)

From table 1 it is evident that the organism "H" is highly fermentative, acid and copious gas being produced in practically all sugars. Growth is very rapid and abundant, less than two hours being needed to obtain a fairly turbid broth culture. The bacterium grows in plain as well as in glucose media, but far more abundantly in the latter. It neither liquefies gelatin in forty days at 37°C. nor peptonizes milk, nor does it attack cellulose. This latter property was tested by the method advocated by Khouvine (1923). A metallic luster appears on the colonies which develop on Endo plates, and the methyl red test is positive. Nitrate reduction is negative and indol is not formed. Cultures grown in glucose broth develop a final hydrogen ion concentration (in twenty-four hours) of pH 4.3. They also give off a very disagreeable odor and tests with lead acetate show the presence of hydrogen sulphide. The organism is remarkably heat resistant, withstanding an exposure to 80°C. for forty-five minutes, although no spores are demonstrable.

The cultures studied show a very interesting and definite pleomorphism, which depends upon the composition of the medium. On glucose agar the bacterium appears as a rather large Gram-negative rod, staining irregularly. If such a culture is transferred to plain agar or broth, it becomes oval shaped, resembling a coccobacillus. This form is maintained until transference to glucose media, when it again returns to a rather large rod, the original form noted in the olive flesh specimen. The morphological changes may occur inside of eighteen hours when the cultures are daily alternated from glucose to plain media.

It is evident that the organism is not a representative of the true coli group. The sugar reactions differ from those of communis, communior and aerogenes. Indol is not produced. Then too, the extreme pleomorphism and low final acidity that are characteristic of the organism under consideration are not common to the better known types of coli. This bacillus will survive heating at eighty degrees for forty-five minutes whereas fifteen minutes at this temperature suffices to kill practically all non-sporulating Gram negative rods. But considering its fermentative ability, its rapidity of growth and the production of H_2S, one feels justified, however, in considering it as one of a species not far removed from the coli-aerogenes group.

TESTS FOR PROPERTY OF PRODUCING FLOATERS

Having obtained this highly fermentative organism, its function in olive spoilage was next determined. Since olives contain mannitol, as well as small amounts of glucose and maltose, and since the organism which has been described is capable of fermenting these carbohydrates, it was reasonable to assume that it might be at least one causative factor of floaters. This assumption was proved to be correct by the following experiments. Uncured ripe Sevillano olives collected directly from the tree, were sterilized by autoclaving in flasks containing sufficient physiological salt solution to allow a covering of at least two inches of fluid. Six to 8 olives were ordinarily placed in one flask. When sterilized the olives sink to the bottom of the container. It is more satisfactory to use uncured rather than cured fruit, since floaters can be produced only occasionally in processed olives. Curing, in most instances seems to change the chemical nature of the carbohydrates, without which fermentation and the consequent production of gas filled pockets does not occur.

Such flasks were inoculated with cultures of organism "H" and incubated, one set at 37°C., one at 28°C., and one at room temperature. The olives had previously been pricked by a sharp needle, that the organism might readily enter the flesh. In twenty-four hours those incubated at 37° were floating upon the surface and showing the typical gaseous blisters. Those at 28° took two to three days to develop blisters, and those at room temperature about the same length of time as those at 28°. This was due possibly to the rather high temperature of the laboratory in which they were kept. Cultures of the infected olives yielded the same organism as had been inoculated. Subsequent tests using olives with unbroken skin showed them equally susceptible to the invasion of the organism. It, therefore, must enter either through the stem opening or through minute invisible holes or pores in the skin. After three or four days these blisters become ruptured and the olives sink to the bottom of the flask. Such breaks in the skin may well serve as a subsequent portal of entry for proteolytic organisms.

BACTERICIDAL EFFECT OF NaCl AND ALKALI

Just how this invasion might be checked or controlled was next considered. The suggestion has been made that pasteurization of the fruit at 175°F. for thirty minutes before curing will temporarily inhibit fermentation if the olives are subsequently cured at minimum temperatures. Our experiments lead us to believe that it is this low temperature processing rather than the pasteurization that retards spoilage. We have spoken above of the actual thermo-stability of this organism so heat does not offer a satisfactory solution of the difficulties, since the temperature which would be necessary to sterilize would soften the olives. Tests were next made as to the concentration of lye or salt which is necessary to kill the organism in culture.

Test tubes containing 8 cc. of varying concentrations of salt in broth, were inoculated with 0.2 cc. of a young broth culture. After twenty-four hours those showing no visible growth were subcultured to broth to determine whether the organisms had been killed or merely inhibited. All concentrations of salt above 5 per cent showed inhibition but subcultures from these into broth yielded growth. A concentration of 10 per cent salt would be necessary if the organisms were to be destroyed but lower concentrations, not less than 5 per cent, could possibly be used to prevent multiplication of the organisms which are on the exterior of the fruit. If they are already in the flesh of the olive gaining entrance by way of the stem canal or through pores in the skin, it is impossible to say, at present, just how far these salt concentrations will act. If, on the other hand, it is found that the bacteria are only on the surface or are introduced with the holding solutions, then a 5 to 10 per cent of salt concentration might considerably lower the incidence of spoilage.

Various concentrations of a staple commercial lye (Hooker's) were next tested. Two-tenths of one percent (0.2 per cent) by weight was found sufficient to kill the organisms in eighteen hours, using 5 cc. of various concentrations of lye made up in broth and 0.1 cc. of a fluid culture of the organism. Incubation at both room temperature and 37° showed the same results. On testing electrometrically 0.2 per cent lye-broth solution, a pH of 12.3 was determined. This alkalinity is far too high to be practicable in olive curing. In tests with pure sodium and potassium hydroxide using the above technique, it was found that it took 0.4 per cent sodium (pH 12.21) and 0.5 per cent of potassium hydroxide (pH 12.26) to sterilize completely.

A GROUP OF ORGANISMS RATHER THAN A SINGLE SPECIES PROBABLY RESPONSIBLE

The organism designated on table 1 as "M" was isolated from another lot of typical "floaters" at a later date than was "H." It gives the same staining and sugar reactions as "H" and does not liquefy gelatin. The colonies give no metallic luster on Endo, but this organism also reproduces typical floaters when inoculated into sterile olives. It produces indol and does not give hydrogen sulphide. But otherwise it is similar in all its reactions, and exhibits the same pleomorphism as the organism we have designated as "H."

It will be necessary to carry on examination of many more lots of diseased olives before definite conclusions can be reached as to just how many different organisms are capable of producing "floaters." It seems evident that more than one species can cause this type of spoilage. The bacterial species thus far encountered, however, exhibit so many similar characteristics that they are apparently very closely related. The culture designated as "F" on table 1 was isolated last year, and it also produces floaters. It differed from the other two in certain sugar reactions, but in staining, pleomorphism, lack of liquefaction of gelatin and other reactions, it was identical. This culture was accidentally destroyed shortly after it was isolated, so that complete comparison was impossible. "H" and "M," however, have been carefully studied and their respective reactions determined by repeated testing. They appear to be more closely allied to the coli group than to any other. True coli, however—both communis and communior being repeatedly tested—did not produce floaters when inoculated into olives, the cultures remaining viable for at least three weeks. Anaerobes have not been isolated from these gas pockets, but the organisms herein described are, as has been demonstrated, quite heat resisting and will grow in partial anaerobiosis.

CONCLUSIONS

1. The predominating organisms present in olives developing blisters or "floaters" during curing are rather large Gram negative rods which show great pleomorphism.
2. These organisms are highly fermentative, but do not liquefy gelatin, peptonize milk, or attack cellulose.
3. More than one species can apparently produce these floaters.
4. These seem closely allied to, but not identical with the colon bacillus.
5. Inoculated into sterile uncured olives, they produce the typical spoilage from which they were isolated.
6. Cultures from the artificially infected olives yield pure cultures of the organisms inoculated.
7. The organism designated as "H" resists 80°C. for forty-five minutes.
8. Ten per cent salt solution is necessary to kill the organisms (twenty-four hours) but concentrations above 5 per cent inhibit the growth.
9. Concentrations of (*a*) commercial lye of 0.2 per cent (pH of 12.5), (*b*) 0.4 per cent NaOH (pH 12.21) or (*c*) 0.5 per cent KOH (pH 12.26) are necessary to sterilize broth cultures.
10. Since sterilization by heat or alkali is not practicable, the best way to attempt to combat this condition that can be offered at present is by frequent thorough sterilization of the vats and curing of the olives at low temperatures. A brine holding solution of at least 5 per cent if not injurious, would probably minimize the danger of spoilage.

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BACTERIA IN THE MUSCULAR TISSUES AND BLOOD OF APPARENTLY NORMAL ANIMALS

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Meat preservation depends, fundamentally, on the inhibition of microörganic growth. Organisms concerned in meat spoilage may enter the product during its handling or may be in the tissues or blood of the live animal. The question of whether or not bacteria are present in the organ tissues of healthy animals at the time of slaughter has a direct bearing upon packing house practice. The literature leaves this problem in an unsatisfactory state. The observations recorded in this paper are part of a series of studies on the bacteriology of meat spoilage carried out in this laboratory under a Fellowship established by Dr. Arthur Lowenstein and in coöperation with the Institute of American Meat Packers.

REVIEW OF THE LITERATURE

A number of investigations to determine whether or not bacteria are normally present in the tissues of apparently healthy animals were reported before 1912, principally by German workers, but very few reports are found in the literature of more recent years. Many of the early workers (e.g., v. Fodor, 1884; Hauser, 1886; Neisser, 1896; Opitz, 1898; and Selter, 1906) believed the tissues of healthy animals to be sterile. Messner (1910) found no bacteria in 145 samples of flesh of "normal" slaughter animals. Zwick and Weichel (1911) examined 77 samples of muscle, liver, spleen, and kidney of cattle and hogs. Of 8 samples from liver 6 were positive, but only 1 muscle sample among the 59 tested contained bacteria. All other samples were sterile. In 1912 Grunt found only 2 per cent of 540 muscle samples to contain bacteria.

Other workers have obtained contrary findings. Thus, Carrière and Vanverts (1899) found a variety of microörganisms in the spleen of healthy dogs, rabbits, and guinea pigs. Ford (1901) examined 96 samples of kidney and liver from 34 rabbits, guinea pigs, cats, and dogs and found bacteria in 70 per cent of the samples. Boni (1901) examined the lungs of 20 hogs for aerobes and found 14 positive (70 per cent), while among 10 lungs of guinea pigs 2 were positive (20 per cent). Quensel (1902) found 30 per cent of 94 normal bronchial lymph nodes from horses, cattle, sheep, and hogs to contain aerobic bacteria; the lungs of 42 "normal" slaughtered animals contained bacteria in 90 per cent of the samples. Saski (1908) examined 8 organs from guinea pigs and mice and isolated 39 strains of bacteria from them, of which 21 were facultative anaerobes, 10 strict anaerobes, and 8 strict aerobes. Conradi (1908) examined 162 samples of muscle, liver, kidney, lung, lymph node, and spleen from 150 slaughtered cattle and hogs, and found that 72 were not sterile (40 per cent). Thirty of these gave anaerobic growth. Wolbach and Saiki (1909) recorded the finding of a new anaerobic spore-bearing bacillus in 21 of 23 livers of healthy dogs. In 1910 Amako found aerobes in 48 samples of 88 (55 per cent) taken from muscle, spleen, kidney, and liver of cattle, and Bierotte and Machida (1910) got a similar percentage of positives (59 per cent with 11 per cent anaerobes) from 54 samples of the same organs of cattle, sheep, and hogs. Bugge and Kiessig (1911) examined 241 muscle samples from 66 head of freshly slaughtered cattle and found aerobes in 36 samples (15 per cent), and in repeated tests, recorded in 1919, they obtained 64 growths from 283 samples (23 per cent).

All of these investigations were made with samples taken from killed animals. Although several investigators have suggested that bacteria probably are present in the tissues of live animals, we have found no reports of work done with samples taken while the animals were alive.

The question naturally arises how bacteria may gain entrance to the body tissues. Those of the above mentioned workers who have expressed their opinion regarding the probable source of the organisms found seem to be agreed that bacteria do not live and propagate in normal tissue *in vivo*. Some (Saski, Conradi, Selter) offer the explanation that bacteria are constantly invading the circulation from the intestinal tract, but are filtered out in a very short time by such organs as lymph nodes, liver, and lungs, and may be withheld in these tissues for some time. Others (Amako, Zwick and Weichel, Bugge and Kiessig, Grunt) think that the presence of bacteria in the tissues of freshly slaughtered animals is due to contamination during the manipulations of slaughtering, such as through the stick wound, and as a result of mechanical pressure on the carcasses.

Bacteria might enter the body tissues from a number of sources, such as the alimentary canal, focal infections, the skin, the urogenital canals, and other mucous membranes. On the basis of many reports (e.g., Wyssokowitsch, 1886; Neisser, 1896; Thöle, 1912; Haas, 1922) we may infer that bacterial invasion from the healthy intestinal tract is not a common occurrence, but that it readily takes place under conditions of more or less severe pathological alterations of the intestinal wall (Waterhouse, 1890; Maklezow, 1897; Buchbinder, 1900; Erkes, 1918). However, it is generally agreed (Welch, 1891; Schott, 1901; Klimenko, 1904; Moody and Irons, 1923) that a perfectly healthy intestinal wall is rare, even in apparently healthy animals, and it is therefore probable that bacterial invasion from the intestinal tract takes place in animals of apparently good health.

The combined experimental and clinical data recorded by a number of investigators (e.g., Rosenow, 1912, 1919; Davis, 1912; Billings, 1914; Irons, 1921, 1924; Haden, 1925) enable us to assume that focal infections offer ample opportunity for the penetration of bacteria into the blood circulation of apparently healthy animals, and the same assumption may hold for other possible portals of entry (Garré, 1885; Manfredi, 1899; Thiele and Embleton, 1913–14; Maxcy, 1919; Strauss, 1924).

EXPERIMENTAL WORK

This work is an outgrowth of attempts to determine the bacterial flora of good and sour hams. In our earlier experiments (Reith, 1926) we found bacteria present in all chilled and non-chilled fresh hams examined from slaughter houses. On the theory that bacteria might enter the hams during the handling of the carcasses on the killing floor, especially during scalding and dehairing, samples of hams were taken from various stages in operation and examined for bacteria. In addition, samples of thigh muscles and blood of live hogs, rabbits, and guinea pigs were examined.

The media employed were those in ordinary use, titrated to pH 7.4. Sugar media were prepared with 1 per cent of test substance. All tubes and flasks of medium, except aerobic slants, were boiled for ten minutes followed by cooling to 40°C. immediately before inoculation. Anaerobic cultures were sealed with a layer of vaseline.

The stages in killing floor operations from which the samples were taken were (*a*) before scalding, (*b*) after scalding, and before dehairing, and (*c*) immediately after dehairing.

Light weight and apparently sound hogs were selected, and 24 hams, 8 from each stage in the operation, were examined. The non-scalded carcasses were cleaned thoroughly with hot water, and the hams were skinned before removal from the carcasses; the scalded carcasses were cleaned and scraped by hand before cutting off the hams. All hams were taken directly to the laboratory. Samples were obtained within one hour after killing. The hams were sectioned at about one to two inches below the symphysis pubis, and the whole cut surface of the upper portion was seared with the direct flame of a Bunsen burner. Suitable sample areas were chosen, the seared crust was removed with sterile instruments from these areas and a new fresh surface seared in the same manner. Approximately 3-gram samples of muscle were then excised aseptically from these twice seared areas and were distributed as follows:

1. One gram portions inoculated into beef heart medium.

2. The remainder ground aseptically with 5 cc. of 0.5 per cent salt solution and distributed, (*a*) a few drops to a blood agar slant, (*b*) 0.5 cc. to each of several glucose agar shakes, (*c*) the remainder heated at 80°C. for ten minutes, then cultured in (1) plain agar slants, (2) glucose agar shakes.

The cultures were incubated at 37°C. and the presence or absence of growth was recorded after three, seven and fourteen days. Results are summarized in table 1 together with the results of our earlier work.

There is a higher percentage of total growth in the samples from the hams taken before scalding, and the lowest percentage is obtained from the hams which had just passed through the dehairing machine. Considering the relatively small number of samples this variation might not be of great significance, yet, the findings do controvert the theory that an increase of bacteria in the hams occurs during the processes of scalding and dehairing. The percentages of growth obtained were considerably higher than those recorded by the German workers. This difference might be explained by the facts that the test animals used by the German workers were chiefly cattle and, more significantly, that their results were based on final readings after one to three days' incubation. Many of our cultures did not show growth until the seventh day, some not before fourteen days, and in one instance anaerobic growth was noted in a beef heart culture only

TABLE 1

Bacteria in hams of slaughtered hogs

SAMPLES TAKEN	NUMBER OF HAMS EXAMINED	NUMBER OF SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
Before scalding	8	39	36 (92%)	20 (51%)
After scalding, before dehairing	8	39	31 (79%)	20 (51%)
Immediately after dehairing	8	41	28 (68%)	22 (54%)
Immediately after evisceration	11	52	43 (83%)	38 (73%)
After forty-eight-hour chill	4	20	16 (80%)	14 (70%)
During cure	3	15	13 (87%)	9 (60%)
Total	42	216	167 (77%)	123 (57%)

after the twenty-second day. Also, the present-day improved anaerobic technic probably tends to increase the number of anaerobic growths.

The distribution of areas from which samples were taken is shown in figure 1, and table 2 gives the relative percentage of growths from these areas.

The figures show that there was no relationship between the number of growths and the depth of area in the ham. If areas 1, 5, and 6 be called deep areas, we have two deep areas from which fewer growths than the average were obtained and one with a larger percentage than the average. Likewise, two of the surface areas (nos. 2, 4) yielded growths above the average and one below.

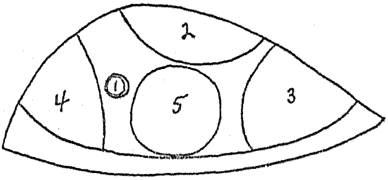

FIG. 1. CROSS SECTION OF HAM 1 TO 2 INCHES BELOW THE SYMPHYSIS PUBIS, SHOWING SAMPLE AREAS

Sample 6 was taken from deep muscles at a 2-inch lower level

TABLE 2

Comparative percentages of growths from the sample areas

SAMPLE AREA	NUMBER OF SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
1	22	15 (68%)	10 (45%)
2	22	17 (77%)	15 (68%)
3	14	13 (93%)	5 (36%)
4	17	13 (76%)	10 (59%)
5	17	12 (71%)	6 (35%)
6	27	25 (93%)	16 (59%)

Live animals

Having found bacteria in hams in all stages of the killing floor operations our next step was to examine muscle and blood samples from living animals. Adult guinea pigs and rabbits, and hogs weighing from 53 to 76 pounds were used. The operations were performed with special precaution against contamination. The instruments were autoclaved for one-half hour at 20 pounds' pressure; the work was done at night to minimize the danger of contamination from dust; the floor and tables of the room were washed with lysol solution; the animals were thoroughly washed with soap and shaved; and the area of operation was swabbed with mercurochrome. The media used had been incubated for four days after sterilization to test their sterility.

The animals were anaesthetised with ether and an incision was made on the inner side of the thigh. Having removed the fascia, a strip of muscle (*M. gracilis*) was cut out and placed in a Petri dish. Approximately 1 gram samples were cultured in each of 3 beef heart tubes and in 3 tubes of broth, in 50 cc. quantities, containing a few grams of ground beef heart. The blood was drawn aseptically directly from the heart and was distributed in 1 cc. quantities to 9, 49, 99, and 299 cc. quantities of sterile broth containing 3 to 5 grams of ground beef heart in each portion the purpose of the beef heart being to promote anaerobic growth.[1] The cultures were incubated at 37°C. and readings made and recorded after two, four, seven and fourteen days. The results are summarized in tables 3 and 4.

The most striking feature of these tables is the high percentage of growth in samples of hog tissues, being 100 per cent from both muscle and blood. It should be noted that the percentage of anaerobic growth from live hog muscle (56 per cent) is practically the same as that from the muscles of slaughtered hogs (57 per cent).

Table 5 shows the percentages of growth in cultures of blood in various dilutions with broth. In the dilution of 1:100 we note the highest percentage of growth, probably because the

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[1] A tube (*A*) containing 45 cc. of broth was inoculated with 5 cc. of blood. After thorough shaking 10 cc. of the broth and blood mixture were transferred to each of tube *B*, containing 40 cc. of broth, flask *C*, with 90 cc. of broth, and flask *D*, with 290 cc. of broth, and 10 cc. were discarded. By this method 1 cc. of blood was contained in each vessel, in dilutions of 1:10, 1:50, 1:100, and 1:300, respectively. The tubes were large test tubes 8 × 1 inch, and the flasks standard Erlenmeyer flasks of 150 and 500 cc. capacity.

TABLE 3

Bacteria in muscular tissues of healthy live animals

ANIMAL	NUMBER	NUMBER OF SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
Hog	6	36	36 (100%)	20 (56%)
Rabbit	6	36	22 (61%)	13 (36%)
Guinea pig	6	36	32 (89%)	20 (55%)
Total	18	108	90 (83%)	53 (49%)

TABLE 4

Bacteria in blood of healthy live animals

ANIMAL	NUMBER	NUMBER OF SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
Hog	6	6	6 (100%)	6 (100%)
Rabbit	6	6	4 (67%)	1 (17%)
Guinea pig	6	6	2 (33%)	1 (17%)
Total	18	18	12 (67%)	8 (44%)

TABLE 5

Growths from blood samples diluted with various quantites of broth

ANIMAL	DILUTION	TOTAL SAMPLES	NUMBER SHOWING GROWTH
Guinea pigs	1:10	6	0
	1:50	6	1
	1:100	6	2
	1:300	6	1
Rabbits	1:10	6	0
	1:50	6	1
	1:100	6	3
	1:300	6	2
Hogs	1:10	6	3
	1:50	6	3
	1:100	6	5
	1:300	6	4
Total	1:10	18	3 (17%)
	1:50	18	5 (28%)
	1:100	18	10 (55%)
	1:300	18	7 (39%)

bactericidal action of the blood is less in this dilution than in any of the lower test dilutions.

Types of bacteria found

Partial identification of the bacteria isolated from the tissues and blood of hogs was attempted on the basis of morphology in the case of the aerobes and of cultural reactions in protein media in the case of the anaerobes. After the original sample cultures had been incubated fourteen days subcultures were made from them in 0.1 per cent glucose agar shakes and on plain agar slants. A portion of the broth of each culture was heated in a water bath for ten minutes at 80°C. and cultured in the same agar media. This method was followed to differentiate between strict aerobes (non-spore-bearers) and strict anaerobes (spore-bearers). Readings were recorded after one, two, three, seven and fourteen days' incubation. The aerobes were then cultured on agar slants and morphological examinations made from smears of twenty-four-hour cultures. The anaerobes were cultured by placing small pieces of agar containing anaerobic colonies in beef heart medium, brom cresol purple milk, and alkaline egg water (Kahn, 1922) and records were made after two, four, seven and twenty-four days' incubation.

One hundred and six aerobic cultures were examined. Twelve gave no growth. Morphological identification of the remaining 94 was made as follows:

55 (59 per cent) staphylococci
21 (22 per cent) bipolar rods
18 (19 per cent) Gram-positive rods
15 (16 per cent) coccus forms other than staphylococci
6 (6 per cent) Gram-negative rods

On the basis of their cultural reactions in the three test media 59 anaerobic cultures obtained from the tissues of the animals were classified roughly as follows:

	Number of cultures
Strongly proteolytic	11
Feebly proteolytic	24
Non-proteolytic	6
No growth, or uncertain reaction	18

We realize that this method of grouping is crude, as more than one type of anaerobe might be contained in the cultured pieces of agar. The reactions in the media used, however, were usually sufficiently characteristic to determine whether the predominating anaerobes present were strongly, feebly, or non-proteolytic.

It has been suggested by several investigators (e.g., Nocard, 1895; Ficker, 1905) that the finding of bacteria in animal tissues is influenced in part by the period of time since the last ingestion of food before the sample is taken. A series of tests were therefore conducted to determine whether or not starvation of thirty-six to forty hours' duration had any bearing on the question.

Assuming that bacteria are present in the muscular and other organ tissues of an animal for a shorter or longer period if they are present in the general circulation, this second series of tests was made on samples of blood from 20 hogs. The method and technic were the same as those employed in the first test. The animals were apparently healthy and of approximately the same size, weighing from 65 to 85 pounds. They were received from the stock yards in two lots, 10 each, and the blood was drawn on the third day after their arrival. The hogs of the first lot were fed as much ground corn as they wanted to eat until the time of the operation. The second lot had one feeding of ground corn immediately after their arrival and no other food until the blood was drawn. These hogs thus had been starved ("empty stomach") for thirty-six to forty hours when the samples were taken. Both lots had plenty of water. The results are given in tables 6 and 7.

At least one sample dilution from each of the 20 hogs showed growth in twenty-four hours (100 per cent). Samples from 9 hogs yielded strict anaerobes (45 per cent). As far as the presence of bacteria is concerned there was apparently no appreciable difference between the samples from the two lots.

It is noted that the two groups of hogs gave considerable variation in the numbers of growth of several types of bacteria, but that the numbers of hogs yielding growth of certain types are almost the same for the two groups of hogs. This is due to the fact that four sample portions from each hog were cultured and that several of the portions from one hog occasionally yielded the same species. The figures in this table, therefore, do not indicate any appreciable difference between the samples of the two lots of hogs.

TABLE 6

Growth from blood samples of well-fed and starved hogs

LOT	DILUTION WITH BROTH	TOTAL SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
Well-fed hogs	1:10	10	8	0
	1:50	10	7	0
	1:100	10	9	2
	1:300	10	10	2
Starved hogs	1:10	10	9	1
	1:50	10	8	3
	1:100	10	10	2
	1:300	10	9	3

TABLE 7

Bacteria from blood samples of hogs, differentiated by morphology and cultural characters

	10 WELL-FED HOGS			10 STARVED HOGS		
	Number of cultures	Number of hogs giving growth	Number of growths	Number of cultures	Number of hogs giving growth	Number of growths
Aerobes	34	10		35	10	
Staphylococci		8	17		6	9
Bipolar rods		6	8		7	10
Coccus forms other than staphylococci		2	3		6	8
Gram-negative rods		2	3		3	7
Gram-positive rods		3	3		3	5
Anaerobes	4	4		9	5	
Strongly proteolytic		1	1		1	2
Feebly proteolytic		1	1		2	2
Non-proteolytic		0	0		2	2
No growth or uncertain reaction		2	2		3	3

The combined results from the blood samples of the 6 hogs of the first series and the 20 hogs of the second series are given in tables 8 and 9.

The dilution of blood samples from hogs giving the highest percentage of growth by the method employed is one between 1:50 and 1:300, probably between 1:100 and 1:300.

The high percentages of cocci and bipolar organisms might suggest invasion from the respiratory tract and the skin. The low percentage of Gram-negative rods should be noted. If invasion from the intestinal tract occurs readily, a high percentage of *B. coli* would be expected. Eleven of the 80 aerobic cultures contained Gram-negative rods, and only 2 of these proved to be *B. coli*, both from the same hog. Mills and Dack (1926) have shown that the rate of destruction of staphylococci in the general circulation of rabbits is lower than that of certain in-

TABLE 8

Growth from blood samples of 26 healthy hogs

DILUTION	TOTAL SAMPLES	NUMBER SHOWING GROWTH	NUMBER SHOWING ANAEROBIC GROWTH
1:10	26	20 (77%)	2 (8%)
1:50	26	18 (69%)	5 (19%)
1:100	26	24 (92%)	6 (23%)
1:300	26	23 (88%)	7 (27%)

TABLE 9

Types of bacteria found in the blood of 26 healthy hogs

	NUMBER OF CULTURES	NUMBER OF HOGS GIVING GROWTH	NUMBER OF GROWTHS
Aerobes	80		
Staphylococci		19 (73%)	33 (41%)
Bipolar rods		15 (58%)	20 (25%)
Coccus forms other than staphylococci		12 (46%)	16 (20%)
Gram-negative rods		6 (23%)	11 (14%)
Gram-positive rods		10 (38%)	10 (13%)
Anaerobes	21		
Strongly proteolytic		3 (12%)	4 (19%)
Feebly proteolytic		5 (19%)	5 (24%)
Non-proteolytic		3 (12%)	3 (14%)
No growth or uncertain reaction		9 (35%)	9 (43%)

testinal bacteria (*B. typhosus, B. sporogenes*), and that it is still lower in a segregated vein, *in vivo*, in the same animal. We should consider the possibility that the resisting forces of the body might have a selective action on various bacteria, and it is conceivable that the generally recognized typical intestinal aerobic organisms may be more readily destroyed in the body than those of the bacterial species which we were able to isolate.

From the 80 sample cultures, all of which gave aerobic growth, only 90 morphologies were observed, indicating a large proportion of pure cultures. This condition suggests a low bacterial count for the samples.

Fifteen of the 26 blood samples gave anaerobic growth (58 per cent). From the point of view of meat preservation it seems important that a variety of anaerobes was isolated from live hogs, namely, some with strong, others with feeble, and still others with non-proteolytic powers. Proteolytic anaerobes, probably, are involved in ham souring, and the fact that they may be present in the hams at the time of killing should turn the attention of the meat packing industry toward the problem of effective inhibition of growth, such as chilling and placing in curing vats as quickly as possible, rather than to elaborate attempts of prevention of more or less harmless contamination from the outside.

We do not assume that bacteria "live" and multiply in healthy tissues. Having invaded the general circulation they are probably filtered out and destroyed, or they might remain viable for a shorter or longer period (Helmholz and Millikin, 1925). Our results, however, show evidence of their presence at the moment our samples were taken from the animals. It should be emphasized that, as compared with the bacterial count of one gram samples of many other foods, bacteria were apparently present in very small numbers in our tissue samples, some of them giving no growth.

Our experiments indicate nothing concerning the portals of entry and the routes of invasion, but it can hardly be doubted that bacteria have ample opportunity to gain entrance to the blood circulation of apparently healthy animals. It is true that many workers have failed to find bacteria in the samples examined, but it should be remembered that failure in isolating bacteria from a given sample is no proof of sterility. Adami (1899) has pointed out that some of the German workers have failed to find bacteria in blood samples probably because these were cultured on solid media and therefore in too high concentration of blood to overcome the bactericidal action. English and French workers, he said, generally have had better success because their cultures were made in sufficient quantities of liquid medium. It is realized, however, that much of the early work from which positive results were obtained was done before modern methods in bacteriology had been introduced, and that further work will be required to determine the source of the organisms present and their relation to environmental conditions.

CONCLUSIONS

1. Cultures of the muscular tissues of slaughtered hogs showed the presence of bacteria in 77 per cent of 216 samples examined; 37 per cent of the samples contained anaerobes.
2. Cultures of the muscular tissues of healthy live hogs, rabbits, and guinea pigs showed the presence of bacteria in 83 per cent of 108 samples examined; 49 per cent of the samples contained anaerobes.
3. Cultures of the blood of healthy live hogs, rabbits, and guinea pigs showed the presence of bacteria in 84 per cent of 38 samples examined; 39 per cent of the samples contained anaerobes.
4. A brief period of starvation of hogs (for thirty-six to forty hours) had no appreciable effect on the presence of bacteria in the blood, nor on the types of bacteria found.
5. Our data show that bacteria may be isolated with considerable regularity from the blood and muscular tissues of healthy live hogs, rabbits, and guinea pigs, as well as of slaughtered hogs.

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A METHOD OF STUDYING THE AVAILABILITY OF SYNTHETIC MEDIA FOR STREPTOCOCCI

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I. INTRODUCTION

The present paper describes a method for the biochemical study of bacteria, more particularly applied to the investigation of synthetic media for steptococci. Streptococci are peculiarly adapted for this study because they are comparatively fastidious and non-sporebearing.

Most of our knowledge relating to the metabolism of streptococci is based on the changes which these organisms produce in conventional media. The constituents of their environment are frequently not definitely known. However, in order to comprehend function fully we must first understand the composition both of the cells and their environment.

Many investigators have made interesting contributions to our knowledge of the reactions of bacteria in synthetic media—that is, in media, the compositions of which are definitely known. Very few (Galimard and Lacomme, 1907; Gordon, 1917; Koser and Rettger, 1919; Lapierre, 1903; Uschinsky, 1893) however, deal with streptococci. The aim, here, is not only to accumulate more data on the availability of synthetic media for streptococci but also to obtain such data under accurately standardized conditions. Special effort has been made carefully to control the details.

II. THE PREPARATION OF SYNTHETIC MEDIA

1. Chemicals. The chemicals used were of purest market grades.

2. Apparatus. Extreme care was taken to insure absolute cleanliness of all apparatus employed.

3. Preparation of the solutions. Except in those cases where the solid substances were very hygroscopic, amounts exceeding 0.003 gram were weighed carefully on a sensitive balance; all the weighed salts of each medium were transferred with a spatula from the weighing bottles to a beaker. Most of this mixture was cautiously tapped into a graduated flask; the remainder was transferred with the aid of H_2O. In the case of hygroscopic substances, liquid compounds, and ingredients contained in the medium in quantities less than 0.003 gram, accurate solutions of convenient concentrations were prepared and the calculated amount pipetted into the volumetric flask. Enough H_2O was added to bring the mixture to within a few cubic centimeters of the mark. The mixture was then boiled for three minutes, cooled to room temperature (23° to 25°C.), made up to volume, glass stoppered, and mixed thoroughly. This procedure was finally chosen as the only one that could be used uniformly for the following reasons: Many of the combinations contained substances in very high concentrations—in some more than that required to make saturated solutions. Others contained very difficultly soluble compounds. Furthermore, boiling at this point insured against marked changes in reaction during sterilization.

4. Titration. In determining hydrogen ion concentrations the colorimetric method was used. The principles involved need not be repeated here; they have been thoroughly described by Clark and Lubs (1917). The chief sources of error, color of medium and turbidity of culture, were obviated by superimposing the color of the medium upon that of the indicator by Walpole's comparator method (Walpole, 1914; Dernby and Avery, 1918).

a. Standard solutions used. M/15 $Na_2HPO_4 \cdot 2H_2O$ – M/15 KH_2PO_4 and M/5 $CH_3COONa \cdot 3H_2O$ – M/5 CH_3COOH. The phosphate mixtures were prepared according to Sörensen's technique (1909) and the acetic acid-sodium acetate mixtures according to Walpole's directions (1914). The accuracy of these standard mixtures was verified by the hydrogen electrode.[1]

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[1] We are indebted to Dr. A. Baird Hastings for aid in this connection.

b. Indicators used. The indicators used were those recommended by Clark and Lubs and manufactured by Hynson, Westcott and Dunning, Baltimore. Table 1 shows the character and concentrations of the indicator solutions.

c. Titration technique. Five cubic centimeters of the medium in the volumetric flask (50 cc.) were measured carefully into a test tube containing 0.15 cc. phenolsulphonephthalein. 2M NaOH or M H_2SO_4 was added very cautiously with a pipette accurately graduated to 0.01 cc. until the solution matched the standard pH 7.9. Then, with the same pipette, the calculated amount of the same alkali or acid was added to the remainder, mixed well and a "check" determination of the reaction made.

TABLE 1

NAME	CONCENTRATION OF INDICATOR	SOLVENT	AMOUNT USED PER 10 CC.	RANGE OF pH
	per cent		*cc.*	
Tetrabromphenolsulphonephthalein (brom phenol blue)	0.04	95 per cent alcohol	0.3	2.8–4.6
O-carbobenzeneazodimethylaniline (methyl red)	0.02	Water	0.5	4.4–6.0
Dibromcresolsulphonephthalein (brom cresol purple)	0.04	95 per cent alcohol	0.3	5.2–6.8
Phenolsulphonephthalein (phenol red)	0.02	95 per cent alcohol	0.3	6.8–8.4

The pH of each medium was determined after sterilization. If it fell outside the range, pH 7.2 to 7.9, readjustments were made with sterile alkali or acid.

The chief point of interest here is that no dilution was made throughout the procedure. This was found to be the most accurate general method because there was a marked lack of uniformity in the buffer values of the solutions. We should guard against the following errors: (1) dilution of the medium to avoid the turbidity or color error, or for economical reasons (so usual in the manufacture of conventional media), (2) titrating comparatively small portions with dilute NaOH (M/20 for example) and then adding the calculated amount of more concentrated alkali to the remainder and (3) titrating aliquot portions with one pipette and adding the calculated amount of the same alkali with a larger pipette in order to save time—though the last is possible with calibrated pipettes if the correction values are taken into account.

It must be mentioned also that the volume of the medium is, obviously, increased by an amount equal to the amount of the alkali or acid added. However, with this method the increase is comparatively slight. Moreover, if the results obtained, are to be used for purposes where the exact concentrations of the constituents must be known, these concentrations may be calculated from the titration data recorded.

TABLE 2

NUMBER OF MINUTES WASHED WITH WATER	AMOUNT OF $BaSO_4$ IN WASH WATER
20	2+
35	1+
50	±
65	±

Note: 2+ = quite marked precipitate.

5. Osmotic pressure. This is a very important consideration (Falk, 1923; Frankel, Barber and Pyle, 1919). Our investigations have not yet brought to light the criteria necessary to determine the optimum osmotic pressure for a given bacterial species.

6. Sterilization. We realized the shortcomings of the various procedures for sterilization. We felt, too, that in the present state of our knowledge there is no ideal method. This conviction is an outcome of many experimental tests. Let us discuss briefly the situation.

a. Sterilization by means of the Berkefeld filter. In these investigations it was of paramount importance to maintain the composition of the synthetic media constant. We have found that it is difficult to wash the Berkefeld filter free from certain substances. For example, the Na_2SO_4, remaining in the filter after it is treated with $KMnO_4$ and $NaHSO_3$, is washed out very slowly. The protocol in table 2 gives the results of one of the many tests we have made.

Although it was possible to test for sulphate, we were unable to devise tests for all substances used in the different media. Furthermore, media containing insoluble substances cannot be sterilized by the Berkefeld filter. Such a situation is met with in the studies of the availability of sulphur for certain bacteria. (Lipman, Waksman and Joffe, 1921; Waksman, 1922a and 1922b; Waksman and Joffe, 1922a and 1922b).

b. Sterilization by preparing concentrated solutions and storing them until sterile. This, of course, is possible only with a limited number of substances. It may work well with strong mineral acids as suggested by Doryland (1916). Satisfactory results are not, however, obtainable with many organic compounds or with substances that are only very slightly soluble. In our studies we have tested solutions (50 per cent) of glucose and sucrose. These did not become sterile even though they were stored for six months. Each showed abundant mold development.

c. Sterilization by heat (Arnold sterilizer and autoclave). Both of these types of heat sterilization result in the decomposition of many substances, the most common examples of which are the carbohydrates. It is very difficult, therefore, to study their availability for bacterial nutrition. After many trials, however, this method seemed in general to be less subject to criticism than the others. We finally sterilized all media in the autoclave at fifteen pounds pressure for fifteen minutes.

III. THE CHARACTER OF THE ORGANISM

1. Source. The cultures from which the test organism was isolated were obtained from root canal infections of human teeth.[2]

2. Preparation of media used in determining the biochemical reactions. a. Sugar-free broth. 75 grams Bacto-veal in one liter of tap water were heated in the Arnold sterilizer for two hours, the coagulum thus formed was allowed to settle to the bottom of the container (this is readily accomplished if the cover is tilted slightly, thereby permitting only very slow cooling), and the infusion was strained through a wire sieve. The hot bouillon easily dissolved the 10 grams of pepton and 5 grams of NaCl that were added at this point. An aliquot part (10 cc.) was cooled and adjusted to pH 7.9. The calculated amount of M NaOH was added to the remainder, and the mixture placed in the Arnold sterilizer for fifteen minutes. Such treatment usually results in an increased acidity, the pH changing to a value between 7.7 and 7.5. This preparation had a final pH of 7.7. The medium was filtered and sterilized in the "Arnold" for thirty minutes on three successive days.

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[2] These cultures were obtained from the late Dr. J. M. Levy and Dr. M. L. Rhein.

We have omitted the treatment with *B. coli-communis*, commonly used to free the infusion from sugar. Our reason for omitting this conventional procedure is this: Muscle tissue, from which Bacto-veal is manufactured, contains only one carbohydrate fermented by streptococci,—a reducing sugar, glucose. Hence, we have tested our infusion with the Fehling-Benedict reagent. Consistently negative results for the presence of glucose were obtained. We recommend this method because it is time saving and economical.

b. Carbohydrate broth. The chief point to guard against in this connection is that a sugar in slightly alkaline solution decomposes readily. This occurs to some extent even at room temperature (23° to 25°C.), but very marked decomposition takes place at the temperature of the Arnold sterilizer or of the autoclave. Avery and Cullen (1919b) suggest that it is best to sterilize a concentrated (20 per cent) aqueous solution of the carbohydrate by boiling (ten to fifteen minutes being sufficient). It seemed to us, however, that sterilization through the Berkefeld filter was the best method for this purpose. The sterile sugar solution was then mixed with sterile sugar-free broth in amounts to make the desired dilution (1 per cent) and the sterility of the resulting mixtures tested by incubating them for eighteen hours at 37°C.

c. Meat infusion agar. The basis of this medium was made by infusing 75 grams of Bacto-veal in 500 cc. of tap water in the Arnold sterilizer for two hours. The steps in the procedure for obtaining the finished bouillon product paralleled those for sugar-free broth. To the infusion was added an equal quantity of 4 per cent agar-agar solution, giving a final concentration of 2 per cent. The whole was sterilized in the autoclave for 45 minutes at 15 pounds pressure.

d. Meat infusion gelatin. Hiss, Zinsser and Russell (1922, p. 152) recipe with the substitution of Bacto-veal for fresh meat.

e. Milk. Hiss, Zinsser and Russell recipe (1922, p. 156).

f. Hiss inulin-serum-water. Hiss, Zinsser and Russell recipe (1922, p. 157).

g. Bile. Ox bile obtained from the slaughter house was sterilized for twenty minutes in the Arnold sterilizer on three successive days.

3. Isolation of strains. The fresh material taken from the root canal was planted on hemolyzed sheep blood glucose veal infusion agar slants (0.2 cc. sterile hemolyzed defibrinated blood plus 0.5 cc. 12 per cent sterile glucose solution for each 6 cc. medium) to which 0.1 cc. sterile glucose veal infusion broth had been added after solidification. After eighteen to twenty hours incubation at 37°C. a loopful was streaked on several whole blood glucose veal infusion agar plates. Single typical colonies fished from these plates were inoculated into glucose infusion broth and examined microscopically.

4. Stock cultures. After isolation, each organism was planted in 2 cc. defibrinated sheep blood, incubated for four hours at 37°C. and then stored in the ice-chest at 10° to 15°C. Later, however, we stored all our cultures in Jablons' liver broth[3] (Jablons and Pease, 1918).

5. Seed cultures. Two days before a specific culture was to be used, a loopful (platinum loop, 4 mm. in diameter) from the stock liver broth cultures was planted in veal infusion broth and incubated for eighteen hours at 37°C. This is designated as the seed culture.

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[3] We have found that streptococci remain viable in this medium for three years without transplant. The detailed results are to be published.

6. Biochemistry of the organisms. a. Reaction on blood media. Various schemes for the classification of the streptococci have been proposed. A consideration of the different suggestions offered in the literature has led us to conclude that the best basis for grouping the streptococci is that depending on the blood reaction.[4] The three types recognized are (1) that which hemolyzes the blood corpuscles (*Streptococcus hemolyticus*); (2) that which produces a green coloration (*Streptococcus viridans*); (3) that whch is indifferent to the presence of blood (*Streptococcus non-hemolyticus*).[4]

TABLE 3

SOURCE OF BLOOD	NUMBER OF HEMOLYTIC STRAINS	NUMBER OF VIRIDANS STRAINS	NUMBER OF INDIFFERENT STRAINS
Man	16	7	47
Rabbit	17	7	46
Sheep	4	16	50
Number of "fast" strains	4	7	40

Note: A "fast" strain is one which reacts alike to the various bloods.

Detailed technique for ascertaining the "blood types:" 0.05 cc. culture from the seed tube was dropped on the blood agar (0.5 cc. blood plus 9.5 cc. agar) in a petri dish and spread over the entire surface with a special smooth glass rod. The depth of the agar was 2 mm. Precaution was taken to have the blood evenly distributed. The results recorded were obtained from the examination of widely separated colonies after twenty-four hours incubation at 37°.

In view of the fact that different laboratories use blood from different animal species, we felt it necessary to find out whether the various strains reacted alike to the bloods most commonly used,—human, rabbit and sheep. As might have been expected, the response of the organisms to the different kinds of erythrocytes was not the same. The actual experimental findings for 70 strains may be summarized as in table 3. This table indicates definitely the probable source of many discrepancies. It indicates, too, that a very decided advance in the systematic study of the streptococci may be made by instituting definite standards of procedure.

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[4] It is interesting to note that while this investigation was in progress the Committee of the Society of American Bacteriologists adopted this criterion as the basis for streptococcus classification. (Bergey's Manual for Determinative Bacteriology, 1923.)

b. Other biochemical reactions. Although our choice of the experimental strain depended almost entirely on the bood reactions, yet a complete characterization of the organism must include the description of its ability to (1) ferment various carbohydrates and related substances; (2) coagulate milk; (3) coagulate inulin-serum-water; (4) liquify gelatin and (5) dissolve in bile.

TABLE 4

STRAIN	pH IN BROTH										REACTION IN			
	Plain	Glucose	Mannitol	Galactose	Lactose	Maltose	Sucrose	Raffinose	Salicin	Inulin	Gelatin	Milk	Bile	Inulin-serum-water
Test organism (1074)........	7.7	4.3	6.5	4.6	5.3	4.3	5.3	6.2	4.6	7.7	0	+	0	0
Control (no growth)........	7.7	7.6	7.6	7.6	7.6	7.6	7.7	7.7	7.7	7.7	0	0	0	0

Note: 0, no reaction; + coagulation.

The test medium (10 cc.) in each case except (5) was inoculated with 0.05 cc. culture from the seed tube. In (5) 0.3 cc. bile was mixed with 3.0 cc. culture. All except (3) and (4) were incubated at 37° for twenty-four hours. (3) was incubated for sixty hours at 37° and (4) at room temperature for twenty-four hours. The results for the experimental organism are presented in table 4.

7. Description of the test organism (1074). The organism used in the present study was isolated in 1920 and its characteristics ascertained. All the tests were repeated for this investigation. It is a "fast" strain of *Streptococcus viridans* showing the characteristics given above.

IV. PREPARATION OF BACTERIAL SUSPENSION USED TO INOCULATE THE SYNTHETIC MEDIA AND METHOD OF TRANSPLANT

1. Medium used to grow the organism employed in the test suspension. The medium employed here was the meat infusion agar described in section III, plus 1 per cent glucose. After being melted, the agar was allowed to cool slowly to 43° to 45°C., thus insuring a minimum volume of water of condensation in the petri dishes after solidification. This is a necessary precaution because in too moist a chamber the growth becomes saturated with moisture. Such a condition is very unfavorable for the purpose at hand because (1) it makes the removal of the bacterial masses from the agar surface very difficult and (2) the small fraction that is obtained is contaminated with diffused substances from the medium.

2. Culture. Two loopfuls were transferred from the stock liver broth culture (described in section III) to fresh liver broth and incubated for sixteen to eighteen hours at 37°, thus producing a culture that contained a "minimum" of degenerated cells. From this, three successive subcultures to glucose Bacto-veal agar were made on three successive days. The last culture served as the seed.

3. Suspension. Two possible procedures for removing the growth from the agar suggested themselves. One method involves the detachment of the growth from the subjacent medium with a bent glass rod, simplifying the removal by washing with physiological NaCl solution and pipetting off the bacterial suspension. It is evident that this is not optimum material for the present study. Much of the soluble material in the medium is washed into the salt solution. Such material may be removed by several washings and centrifugations, each time discarding the supernatant fluid. By so doing, however, the diffusible bacterial constituents are continually being drawn upon.

The other method seems far superior for providing a pure mass consisting only of "intact" bacterial structures. This process is simpler and involves fewer manipulations than the first. The smoothed end of a microscope slide (spatula) is held lightly but firmly on the agar surface and rotated slowly clockwise. At the same time the dish is turned slowly counterclockwise. The growth is thereby gathered on the slide, and is very easily tapped off into a sterile petri dish containing physiological NaCl solution.

In order to show definitely the method best suited for our purposes, tests were made on:

a. The supernatant NaCl solution after centrifugation of the bacterial suspension, obtained by removing the growth from the agar with NaCl solution.

b. The supernatant fluid after centrifugation of the bacterial suspension in distilled H_2O, obtained as in (*a*) with distilled H_2O substituted for NaCl solution.

TABLE 5

TEST MATERIAL	TEST PERFORMED			
	Chloride (with silver nitrate)	Phosphate (with ammonium molybdate)	Protein or protein split products (with biuret reagent)	Glucose (with Benedict reagent)
(a)		++++	++++	++++
(b)	++++	++++	++++	++++
(c)		++++	++++	++++
(d)	++++	++++	++++	++++
(e)		±	0	0
(f)	±	±	0	0
(g)	0	0	0	0
(h)	0	0	0	0

c. The NaCl solution after being applied to the fresh agar surface and manipulated as though growth was actually being removed.

d. The distilled H_2O wash obtained under the same conditions as in (*c*).

e. The supernatant fluid from a bacterial suspension in a NaCl solution, the bacteria having been removed from the agar by the spatula method.

f. The supernatant fluid from a bacterial suspension in distilled H_2O, the growth having been removed as in (*e*).

g. The NaCl solution used to wash a glass slide manipulated over a fresh agar surface as though growth was being removed.

h. The distilled H_2O wash obtained as in (*g*).

Table 5 gives the results of qualitative tests for phosphate, chloride, protein or protein split products and glucose. The letters in parentheses refer to the several descriptions listed above.

The experiments show that (1) substances are transferred from the medium to the wash solution; (2) bacteria suspended in distilled H_2O or NaCl solution may give up some of their substance to the surrounding liquid; (3) none of the most concentrated substances are removed from the medium by the mere application of a smooth spatula. This evidence points definitely to the second method as the superior one. We have, therefore, chosen it for our work.

The suspension prepared as described above was diluted to match a stock suspension of streptococci standardized so that each cubic centimeter contained two billion organisms. One-hundredth cubic centimeter of this constituted the "test" dose for 1 cc. of medium.

4. Method of transplant. After a given medium was sterilized, seven 1 cc. portions were pipetted into test tubes. One of these was used to determine the pH; another was inoculated with the test dose (first transplant) and incubated at 37°C. The remaining five tubes, to be used for successive transplants on the five succeeding days, were covered with towels previously soaked in 5 per cent phenol solution. In order to insure against evaporation from these tubes the towels were kept moist.

Twenty-four hours after the inoculation into the first tube of synthetic medium a loopful (6 mm. diameter) was transferred from the first transplant to a second tube of synthetic medium (second transplant) and a loopful (3 mm. diameter) to glucose veal infusion agar (subculture), the composition of which was the same as that used to grow the organism contained in the test dose. The transplants to agar were used to test the presence of live organisms in the synthetic medium culture. No clouding of the synthetic medium occurred, thus indicating the absence of rapid propagation. The amount of growth on the agar was recorded after twenty-four hours incubation at 37°. This gave the result for the first subculture of the first transplant—forty-eight hours after inoculation. The second transplant was incubated and treated the following day in the same manner as the first transplant. At the same time, another transfer was made from the first transplant to glucose agar. This procedure was continued for six transplants. The subcultures from each transplant numbered one more than the one immediately following. Table 6 presents a sample protocol for two media.

The dates immediately under the words "transplant" are those on which the inoculations in the synthetic media tubes were made; those below the words "subcultures" designate the days on which were read the results of the transfers from synthetic medium to glucose agar. The results indicate comparative estimates of the quantity of growth on glucose agar.

TABLE 6

NUMBER OF MEDIUM	FIRST TRANSPLANT, MAY 12, 1924, SUBCULTURES						SECOND TRANSPLANT, MAY 13, 1924, SUBCULTURES					THIRD TRANSPLANT, MAY 14, 1924, SUBCULTURES				FOURTH TRANSPLANT, MAY 15, 1924, SUBCULTURES			FIFTH TRANSPLANT, MAY 16, 1924, SUBCULTURES		SIXTH TRANSPLANT, MAY 17, 1924, SUBCULTURES
	May 14	May 15	May 16	May 17	May 18	May 19	May 15	May 16	May 17	May 18	May 19	May 16	May 17	May 18	May 19	May 17	May 18	May 19	May 18	May 19	May 19
461	3+	3+	+	2+	0	0	4c	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5bp	4+	4+	4+	4+	3+	1c	2+	3+	3+	2+	2+	2c	2c	+	+	0	0	0	0	0	0

Note: c = colonies; 4+ = maximum growth; 0 = no growth.

V. AVAILABILITY OF SYNTHETIC MEDIA FOR STREPTOCOCCI

1. Review of literature. Synthetic media, that is, media, the compositions of which are definitely known, have been employed in various researches on the nutrition and metabolism of microorganisms. Classified on the basis of the organism studied, these investigations may be tabulated as follows:

a. Molds and yeasts. Naegeli (1882), Czapek (1901; 1903), Dox (1912), Currie (1917), Pieper, Humphrey and Acree (1917), Fulmer, Nelson and White (1923).

b. Colon-typhoid group of bacteria. Uschinsky (1893), Capaldi and Proskauer (1896), Wheeler (1905), Galimard and Lacomme (1907), Dolt (1908), Bainbridge (1911), Keyes and Gillespie (1912), Sasaki (1914), Sperry and Rettger (1915), Rettger, Berman and Sturges (1916), Bengis (1916), Zunz and György (1916), Gordon (1917), Clark and Lubs (1917), Ayers and Rupp (1918a and b), Frankel, Barber and Pyle (1919), Kisch (1919), Koessler and Hanke (1919), Koser and Rettger (1919), Braun and Cahn-Bronner (1921), Scales (1921).

c. Cholera. Uschinsky (1893a and b), Maassen (1894), Voges (1894), Galimard and Lacomme (1907), Gordon (1917), Frankel, Barber and Pyle (1919), Frankel and Pyle, (1919), Kisch (1919), Koser and Rettger (1919).

d. Pigment bacteria. Fermi (1892), Jordon (1899), Samkow (1904), Sullivan (1905–06), Frouin and Ledebt (1912), Franzen and Egger (1914), Blanchetière (1917).

e. Sulphur bacteria. Beijerinck (1895a, b and c, 1900, 1904), Nathanson (1901–02), Van Delden (1904a and b), Lipman, Waksman and Joffe (1921), Trautwein (1921), Waksman (1922 a and b), Waksman and Joffe (1922a and b).

f. Nitrogen bacteria. Winogradsky (1890a and b, 1894, 1896a and b), Winogradsky and Omelianski (1899), Omelianski (1899a and b), Omelianski and Sserwerowa (1911), Gibbs (1919), Hunter (1923), Meek and Lipman (1923).

g. B. diphtheriae. Uschinsky (1893a and b), Hadley (1907).

h. B. tuberculosis. Kühne (1893), Proskauer and Beck (1894), Frouin (1912), Armand-Delille et al (1913), Lockemann (1919), Borrel (1922), Long (1922).

i. Cocci. Uschinsky (1893b), Lepierre (1903), Galimard and Lacomme (1907), Bainbridge (1911), Sperry and Rettger (1915), Rettger, Berman and Sturges (1916), Gordon (1917), Frankel, Barber and Pyle (1919), Koser and Rettger (1919).

This is not a complete list; it gives, however, the typical researches in the field.

2. Composition of media and results showing their availability. a. Types of media. Instead of devising new combinations of nutrients, we used, in the greater number of the 671 media tested, those described by previous workers. This plan was followed because it was hoped to correlate more readily our findings with those of these earlier investigators. It was impossible to duplicate a number of media because (1) very few recipes are described in any detail and (2) in some cases the precise names of the chemicals used are not recorded.

The chief points to be noticed in the present work are: (1) the description of all ingredients was recorded in full; (2) whenever reactions were adjusted with $NaHCO_3$, Na_2CO_3, K_2CO_3, the amounts of these substances were accurately determined and recorded; (3) aspartic acid was substituted for asparagin or sodium asparaginate (Clark and Lubs, 1917);(4) the term "trace" so often used to designate small quantities is assigned a definite value: 0.1 cc. of a 0.1 per cent solution or of a 0.1 per cent suspension (in cases of slightly soluble substances) per 100 cc. of medium; i.e., 0.0001 per cent; (5) the numbers of the media given in the first columns of the tables are the authors' serial numbers.

b. Media and results. The 671 mediawith their corresponding results were originally listed in a series of thirty-four protocols. In general, each consisted of media suggested by one investigator or a group of investigators using related media. However, because a journal cannot afford space for so much detail we are presenting three series as illustrations (see tables 7 to 9).

c. Reading of results. The results recorded in the tables consist only of those necessary to show the comparative values of the various media. The symbols, 4+, 3+, 2+, +, several colonies, c, and 0, are used to indicate the amount of growth; 4+denotes the maximum. Therefore, the records for the subcultures of the last four transplants were omitted from the protocols. However, the tests on each medium included six transplants. The one from the test suspension to the synthetic medium is designated as the first transplant; the five others are successive transplants from synthetic medium to synthetic medium. The subcultures from each transplant numbered one more than the one immediately following; thus, there were six subcultures from the first transplant, five from the second, four from the third, three from the fourth, two from the fifth and one from the sixth (see table 6).

TABLE 7

Series 1

NUMBER OF MEDIUM	REFERENCE	NaOH 2.123 M		PERCENTAGE COMPOSITION													RESULTS OF GROWTH										
																	First Transplant Subcultures						Second Transplant Subcultures				
		Cubic centimeter per 5 cc. before sterilization	Cubic centimeter per 5 cc. after sterilization	NH_4 lactate	Aspartic acid	Glucose	Lactose	Malic acid	Maltose	Sucrose	$CaCl_2$	$MgSO_4 \cdot 7H_2O$	K_2HPO_4	Na_2CO_3	NaCl	$Na_2HPO_4 \cdot 12H_2O$	1	2	3	4	5	6	1	2	3	4	5
510	Frankel and Voges, 1894	0.27			1.0			0.7			0.001	0.2		0.25		0.2	3+	+	0	0	0	0	0	0	0	0	0
511	Frankel and Voges, 1894	0.31	0.004		1.0			0.7		1.5	0.001	0.2		0.25		0.2	0	0	0	0	0	0	0	0	0	0	0
512	Frankel and Voges, 1894	0.29	0.012		1.0		1.5	0.7			0.001	0.2		0.25		0.2	3+	3+	2+	2+	2+	+	+	3c	0	0	0
513	Frankel and Voges, 1894	0.29	0.015		1.0			0.7	1.5		0.001	0.2		0.25		0.2	3+	3+	3+	0	0	0	2c	0	0	0	0
514	Frankel and Voges, 1894	0.30	0.008		1.0	1.5		0.7			0.001	0.2		0.25		0.2	3+	3+	3+	0	0	0	4c	0	0	0	0
595	Frankel and Voges, 1894	0.01		0.6	0.4								0.2		0.5		2+	2+	2+	2+	+	+	0	0	0	0	0

TABLE 3

Series 2

NUMBER OF MEDIUM	REFERENCES	NaOH 2.123 M		PERCENTAGE COMPOSITION												RESULTS OF GROWTH										
		Cubic centimeter per 5 cc. before sterilization	Cubic centimeter per 5 cc. after sterilization	Alanine	Aspartic acid	Glycocoll	Leucine	Tyrosine	Urea	Ca-glycero-PO_4	Glycerol	$MgSO_4 \cdot 7H_2O$	KH CO_3	Na_2 CO_3	NaCl	First Transplant Subcultures						Second Transplant Subcultures				
																1	2	3	4	5	6	1	2	3	4	5
70	Dolt, 1908			0.2			0.75	Tr.		0.02	1.5	0.05		0.024	0.005*	+	0	0	0	0	0	0	0	0	0	0
146	Galimard and Lacomme, 1907									0.2	1.5	0.05	0.03		0.5	0	0	0	0	0	0	0	0	0	0	0
526	Hadley, 1907	0.006				1.0				0.2	1.5	0.05	0.03		0.5	2+	2+	2+	0	0	0	0	0	0	0	0
527	Hadley, 1907						1.0			0.2	1.5	0.05	0.03		0.5†	3+	3+	3+	0	0	0	2+	3+	0	0	0
528	Hadley, 1907	0.004						1.0		0.2	1.5	0.05	0.03		0.5	3+	0	0	0	0	0	0	0	0	0	0
529	Hadley, 1907	0.09	0.015		1.0					0.2	1.5	0.05	0.03		0.5	0	0	0	0	0	0	0	0	0	0	0
530	Hadley, 1907								1.0	0.2	1.5	0.05	0.03		0.5	0	0	0	0	0	0	0	0	0	0	0
531	Hadley, 1907	0.004					0.1	0.9		0.2	1.5	0.05	0.03		0.5	+	0	0	0	0	0	0	0	0	0	0
532	Hadley, 1907	0.003				0.5			0.5	0.2	1.5	0.05	0.03		0.5	2+	+	0	0	0	0	0	0	0	0	0
76	Lepierre, 1903									0.2		0.05	0.1		0.5‡	0	0	0	0	0	0	0	0	0	0	0

* Dolt's, adaptation from Galimard and Lacomme.

† Natural Leucin was used.

‡ Lepierre used also a glycoprotein.

Tr. = trace.

TABLE 9

Series 3

NUMBER OF MEDIUM	REFERENCE	NaOH 2.123 M		PERCENTAGE COMPOSITION														RESULTS OF GROWTH										
		Cubic centimeter per 5 cc. before sterilization	Cubic centimeter per 5 cc. after sterilization	$(NH_4)_2HPO_4$	$(NH_4)_2SO_4$	Aspartic acid	$Ca_2H_2(PO_4)_2 \cdot 2H_2O$	M Citric acid	Glycerol	Mg_3 citrate	$MgHPO_4 \cdot 3H_2O$	$MgSO_4 \cdot 7H_2O$	$M/_3\ H_3PO_4$	K_2HPO_4	KH_2PO_4	$Na_2HPO_4 \cdot 12H_2O$	$NaH_2PO_4 \cdot 4H_2O$	$M/_2\ H_2SO_4$	First Transplant Subcultures						Second Transplant Subcultures				
																			1	2	3	4	5	6	1	2	3	4	5
100	Lockemann, 1919	0.215				0.529		1.0	2.0		0.493		1.0	0.018		0.036		1.0	4+	4+	4+	4+	4+	4+	2+	+	+	+	+
101	Lockemann, 1919	0.214				0.5			2.0	0.25		0.06			0.4		0.3		3+	4+	4+	4+	3+	3+	0	0	0	0	0
102	Lockemann, 1919	0.213				0.529		1.0	2.0		0.493		1.0	0.035				1.0*	4+	4+	4+	3+	3+	4+	0	3c	+	+	5c
285	Lockemann, 1919			0.6					4.0			0.6							0	0	0	0	0	0	0	0	0	0	0
286	Lockemann, 1919	0.08		0.6					4.0			0.6					0.3		4+	4+	4+	2+	3+	2+	2c	3c	2c	6c	0
287	Lockemann, 1919	0.145			0.5				2.0	0.25		0.1			0.4		0.3	†	4+	2+	3+	3+	3+	2+	0	0	0	0	0
289	Lockemann, 1919	0.2				0.5		1.2	2.0		0.4		2.5		0.025	0.025			4+	4+	4+	3+	4+	4+	+	2+	+	+	+
290	Lockemann, 1919	0.24	0.004			0.5	0.025	1.2	2.0		3.75		2.5		0.025	0.025		‡	4+	4+	4+	4+	4+	0	+	5c	3c	0	0
291	Lockemann, 1919	0.21				0.5	0.025	1.2	2.0		0.375		2.5						4+	4+	4+	4+	4+	0	+	+	+	+	0
292	Lockemann, 1919	0.275				0.5	0.29	1.2	2.0				2.5		0.025	0.025			4+	3+	4+	4+	4+	+	+	0	0	0	0
293	Lockemann, 1919	0.185				0.529		1.0	2.0		0.493		2.0			0.072		1.0	4+	4+	1+	4+	4+	+	2+	2+	2+	+	0
284	Lockemann, 1919			0.6					4.0									§	0	0	0	0	0	0	0	0	0	0	0

* Lockemann used 2.0 per cent M H_3PO_4.

† Original contains no $MgSO_4$.

‡ Original uses only 0.375 $MgHPO_4$.

§ This is Lowenstein's medium.

TABLE 10

Summary of data

ESTIMATE OF GROWTH	FIRST TRANSPLANT SUBCULTURES						SECOND TRANSPLANT SUBCULTURES					THIRD TRANSPLANT SUBCULTURES				FOURTH TRANSPLANT SUBCULTURES			FIFTH TRANSPLANT SUBCULTURES		SIXTH TRANSPLANT SUBCULTURES
	1	2	3	4	5	6	1	2	3	4	5	1	2	3	4	1	2	3	1	2	1
4+	103	66	29	19	11	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3+	192	103	80	56	33	23	0	4	2	1	0	0	0	0	0	0	0	0	0	0	0
2+	85	98	80	61	52	29	6	6	2	1	1	0	0	0	0	0	0	0	0	0	0
1+	52	45	42	36	41	31	81	34	27	25	21	0	0	1	1	0	0	0	0	0	0
Several colonies	9	16	13	5	8	4	72	44	31	11	8	1	1	0	0	0	0	0	0	0	0
Total number of media showing growth.....	441	328	244	177	145	93	159	88	62	38	30	1	1	1	1	0	0	0	0	0	0
Percentage of media showing growth.....	66.5	49.7	39.3	28.6	22.0	14.1	25.6	13.3	9.4	5.7	4.5	0.1	0.1	0.1	0.1	0	0	0	0	0	0

VI. SUMMARY

This investigation offers a technique for studying the availability of synthetic media for streptococci. The principles and procedures discussed may be applied to the study of synthetic media in general. Details are given regarding (1) the preparation of media, (2) the determination of the character of the organism, (3) the preparation of the bacterial suspension used to inoculate the synthetic media, (4) the method of transplanting to synthetic media and (5) the method of reading the results.

Of the 671 media studied, 441 permitted the streptococci to remain viable through one or more transplants. While it would seem from this, perhaps, that it is a comparatively simple matter to manufacture synthetic mixtures which will permit the growth of these organisms, our results indicate a marked deficiency in all of these media. The significant details may be summarized as in table 10.

The figures in table 10 indicate: (1) a decrease in the per cent of media showing living organisms in successive transplants; (2) a very marked falling off in the number of "positives" in the successive subcultures of the first transplant series; (3) a change that is not so abrupt in the second transplant series; (4) only one medium that shows growth in the third transplant series and (5) no medium showing growth in the fourth, fifth or sixth transplants.

These media do not permit the physiological functions of this organism to continue normally, hence propagation decreases and finally ceases. Continued growth in the first two transplants may be due either to the small reserve store of necessary nutrients or to the transfer of small amounts of some of the constituents from the conventional medium on which the test culture was amassed—this despite our extreme care to obtain a pure suspension. The amount of substance so transferred must be that adhering to the bacterial bodies.

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STUDIES IN MICROBIC HEREDITY

XI. THE GENETIC ORIGIN OF STAPHYLOCOCCUS ALBUS AND AUREUS FROM COMMON ANCESTRAL STRAINS

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PURPOSE OF THE STUDY

It is not the primary purpose of this paper to classify staphylococci but rather to obtain some clue as to the genetic origin of numerous types whose heterogeneity has helped to baffle those who have attempted to make satisfactory classifications. Incidentally, the reason for the mixed character of the group may become more apparent and the fundamental biological relationships of its individual members be somewhat clarified.

Although our approach to this subject has a serological basis, the genetic significance of the conclusions will have as their background Study III (Mellon, 1926) of this series in which serologic and cultural differences appear as the result of definite variability experiments. It has been shown in this study that from an organism possessed of two or more antigenic groups new individuals may be dissociated, having but one of these groups dominant. In short, the genetic origin of group and specific agglutinogens has been indicated, with and without change of cultural or morphologic character.

SOURCE OF MATERIAL

Although many of the cultures were obtained largely from diverse pathological conditions, the majority came from cases of *impetigo contagiosa bullosa* in whose epidemiology we were interested. Particularly, were we interested in isolating an epidemic type, but in this we were not successful (Mellon, 1925). A most

TABLE 1

Showing the agglutination reactions of the majority of the strains studied (40)

PIGMENT	SOURCE	NUMBER	MANNITOL	AUREUS—NO. 635				ALBUS—NO. 644				ALBUS—NO. 1 (GALL BLADDER)				NORMAL			NaCl SOLUTION	DATE
				1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:20	1:40	1:80		
White	Gall-bladder	1-21	–	2+	1+	1+	±	C–	C–	C–	C–	C	C	C	3+	1+	0	0	00	2/6
White	Gall stone	192-21	–	1+	0	0	0	C	C	C		0	0	0	0	1+	1+	0	00	2/6 and 6/12
White	Gall-bladder	470-22	–	2+	±	0	0	C	C–	C–	C–	C	C	C	2+	1+	0	0	00	2/13 and 9/24
White	Gall-bladder	891-22	–	1+	±	0	0	C	C	C	C	C	C	C	C	C	1+	0	00	2/18
White	Breast milk	644-23	–	0	0	0	0	C	C	C	C	C	C	C	C	0	0	0	00	1/18
White	Breast milk	646-23	–	0	0	0	0	3+	3+	3+	2+	C	C	C	C	1+	1+	0	00	2/15
White	Skin	657B-23	–	0	0	0	0	2+	2+	1+	0	C	C	1+	0	0	0	0	0 00	2/15 and 3/9
White	Breast milk	659-23	–	2+	2+	1+	0	3+	3+	3+	3+	3+	C	C	C–	3+	3+	1+	00	2/15
White	Breast milk	669-23	–	0	0	0	0	0	0	0	0	C	C	3+	1+	1+	0	0	00	2/21
White	Nose culture	820-23	–	±	0	0	0	3+	3+	1+	1+	C	C	C	C	0	0	0	00	3/9 and 6/29
White	Urine culture	836-23	–	1+	0	0	0	C–	2+	2+	0					1+	0	0	00	3/9
White	Breast milk	136-24	–	1+	1+	1+	0	C	C	C	1+	C	2+	1+	0	2+	±	0	00	2/21
White	Mouth culture	164-24	–	1+	1+	±	0	C	C	C	C					1+	0	0	00	3/5 and 6/16
Orange	Gall-bladder	11.1-21	+	C	C	C	C–	1+	1+	±	0					1+	1+	0	00	1/18 and 5/22
Orange	Furuncle	180-23	+	C	C	C	C	1+	±	0	0					0	0	0	00	3/2
Orange	Osteomyelitis	404-23	+	C	C	C	C	2+	2+	0	0					0	0	0	00	3/9
Orange	Breast milk	630-23	+	C	C	C	C	0	0	0	0	1+	1+	0	0	1+	1+	1+	00	1/18

Orange	Impetigo	636-23	+	3+	C	C	C	1+	1+	1+	±	0	0	0	0	±	0	0	00	2/21
Orange	Breast milk	639-23	+	C	C	C	C	0	0	0	0	0	0	0	0	0	0	0	00	1/28
Orange	Eye exudate	657A-23	+	C	C	C	C	0	0	0	0	0	0	0	0	0	0	0	00	2/6
Orange	Eye exudate	658-23	+	C	C	C	C	1+	1+	±	0	0	0	0	0	0	0	0	00	2/6
Orange	Impetigo	664-23	+	C	C	C	1+	1+	1+	0	0		0	0	0	0	0	0	00	2/18
Orange	Impetigo	725-23	+	C	C	C	C	2+	2+	1+	0	1+	1+	0	0	2+	±	0	00	3/2
Orange	Osteomyelitis	856-23	+	3+	C	C	C	3+	2+	2+	0					2+	2+	0	00	3/2 and 6/16
Orange	Breast abscess	26-24	+	C	C	C	C	1+	1+	0	0					±	0	0	00	3/2
Orange	Impetigo	32-24	+	C	C	C	C	2+	2+	1+	0	2+	2+	0	0	2+	0	0	00	3/2
White	Gall-bladder drainage	39-24	+	C	C	C	C	0	0	0	0	0	0	0	0	0	0	0	00	2/21
Orange	Empyema	45-24	+	C	C	C	C	1+	1+	1+	0	2+	2+	1+	0	1+	1+	0	00	1/28
Orange	Impetigo	96-24	+	C	C	C	3+	2+	0	0						1+	±	0	00	3/2
White	Abscess	108-24	−	C	C	C	C	2+	1+	0	0					C	3+	1+	00	6/26
Orange	Impetigo	132-24	+	3+	C	C	C	2+	1+	±	0					1+	0	0	00	3/5
Orange	Conjunctivitis	153-24	+	C	C	C	2+	0	0	0	0					0	0	0	00	3/5
Orange	"Stitch" abscess	162-24	+	C	C	C	C	0	0	0	0	0	0	0	0	0	0	0	00	3/2
Orange	Abscess	169-24	+	C−	C−	C−	C−	1+	1+	1+	1+					0	0	0	00	6/26
Orange	Impetigo	173-24	+	C	C	C	C	1+	1+	1+	0					0	0	0	00	3/5
Orange	Impetigo	175-24	+	C	C	C	C	1+	±	±	0					0	0	0	00	3/5
Orange	Impetigo	213-24	+	2+	C	C	0	0	0	0	0	0	0	0	0	0	0	0	00	3/2
Orange	Impetigo	214-24	+	2+	C	C	C	0	0	0	0	0	0	0	0	0	0	0	00	3/2
Orange	Impetigo	215-24	+	2+	C	C	C	1+	±	±	0	0	0	0	0	0	0	0	00	3/2
Orange	Impetigo	217-24	+	C	C	C	C−	0	0	0	0	0	0	0	0	0	0	0	00	3/2

interesting anomalous group came from pathological conditions in the gall-bladder, the flora of which has been subjected to special study. The remainder were obtained from miscellaneous lesions. The total of the series was 58 strains, which in a preliminary way had been tested on mannitol broth with the idea of determining whether this character could be correlated with pigment formation. If so, it would form a ready method of recognizing aureus strains temporarily non-pigmented (see table 1).

A COMMON ANCESTOR STRAIN FOR AUREUS AND ALBUS STRAINS

It was our good fortune to encounter a strain made up apparently of at least three distinct antigenic groups. One of these groups was serologically albus; the others serologically aureus. The strain itself, no. 644, was never pigmented while under our observation. The cardinal value of this strain for our purpose is illustrated by the observations of Study III of this series (1926), which shows that strains having specific agglutinogens may be dissociated from a parent strain containing both group and specific agglutinogens. The presumption is strong, therefore, that the same thing holds for the staphylococci. Moreover, the common ancestor strain offers a plausible explanation for the diversity among these cocci which has caused certain workers to abandon efforts at satisfactory classification. The bearing of this proposition will become clearer in light of the serologic data.

EXPERIMENTAL

Technique. Immune sera were prepared by immunization of rabbits with killed cultures of strains nos. 644, 635 and 1. Nos. 644 and 1 were non-pigmented strains not fermenting mannitol (*S. epidermidis* according to Bergey). The strains were purified beyond question before inoculation. The first dose consisted of 0.5 cc. of the organisms suspended to a turbidity of one billion per cubic centimeter. Five doses, gradually increasing up to 1 cc. of a two billion suspension, were given at three- to four-day intervals. One week after the fifth inoculation the animals were bled from the heart. The day following the bleeding a sixth dose was given and after another week the animal was bled again.

Antigens were prepared after the method of Hooker. Four-day-old agar cultures were used and the growth washed off with 0.1 per cent formaldehyde in 0.85 per cent NaCl solution. The suspensions were allowed to stand from three to four days, washed, suspended in fresh formolized salt solution, filtered and brought to a turbidity of two billion per cubic centimeter.

Dilutions of serum of 1:25, 1:50, 1:100 and 1:1200 were used in 0.5-cc. amounts. An equal amount of antigen was added to each dilution tube, doubling the final dilution in each tube. No attempt was made to secure a serum of very high titer, nor, to determine the titer of a serum except for the homologous antigen. For the homologous antigen the titer was at least 1:800. Controls in normal serum in final dilutions of 1:20, 1:40 and 1:80 were used, as well as salt solution controls. The earlier set-ups were incubated for two or three hours at 37°C. and then placed in the icebox over night. The results were so unsatisfactory that the tests had to be repeated and for all later work the tubes were incubated at 55°C. for sixteen hours. Second readings were made 24 hours later.

For the absorption of agglutinins the following procedure was used. A twenty-four-hour culture of the organism was emulsified in a small volume of 0.85 per cent NaCl solution, heated to 56°C. for twenty minutes, then centrifuged at high speed until clear. To the sediment thus obtained a 1:10 dilution of the serum was added. The suspension of bacteria in diluted serum was incubated at 37° for two hours and held in the ice box over night, centrifuged until clear, the serum pipetted off and added to another heavy sediment of organisms precipitated as before and the absorption repeated, after which agglutination tests were made.

SEROLOGIC DATA

From table 2 it is clear that strain no. 644, although growing white and not fermenting mannitol, contains a pigmented subgroup which is reciprocal serologically with the antigen of no. 635. The reactions here are precisely those of group and specific agglutinins between any two strains, strain no. 635 being a minor agglutinogen in the complex antigen of no. 644. We do not expect no. 644 antigen to agglutinate noticeably in the serum of no. 635 nor to absorb antibodies from it, while we do expect no. 635 antigen to absorb its own antibodies from serum no. 644 but to leave untouched the group antibodies of no. 644. This is exactly what happened.

TABLE 2

Agglutination reactions of strain no. 635

ANTIGEN	NO. 635 (AUREUS)									NO. 644 (WHITE)				NO. 1 (WHITE)				NORMAL SERUM				NaCl
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:20	1:40	1:80	1:160	
635	C	C	C	C						C	C	C	C	C–	C–	C–	C–	1+	1+	1+	O	–
*	C	C	C	C	C	C	C	C	C													
644	O	O	O	O						C	C	C	C									

ANTIGEN	NO. 635A635†				NO. 635A644				NO. 644A644				NO. 644A635				NaCl
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	
635	2+	1+	1+	1+	C	C	C	C	1+	O	O	O	O	O	O	O	00
644									O	O	O	O	C	C	C	C	00

* Means the second or overnight reading.

† Means that no. 635 serum is absorbed (A) by no. 635 antigen.

Strains nos. 597, 666 and 41 behaved as did no. 635 when run against the three antisera. They all ferment mannitol and are pigmented. Pigmented strain no. 645 represents a subgroup of no. 635 inasmuch as it only removed part of the agglutinin from serum no. 635 on absorption.

Strain no. 744 did not ferment mannitol and was non-pigmented, yet it agglutinated no. 635 serum strongly. Its reaction with serum no. 644 and no. 1 was not complete. It is entirely conceivable that such a strain was dissociated from a no. 644 type with its fermentation of mannitol temporarily in abeyance. Certain of our experimentally dissociated strains have exhibited just such characters.

Reference to table 1 shows clearly that practically all of the pigmented strains agglutinated in no. 635 antiserum but practically not at all in no. 644 serum. Since a pigmented agglutinogen exists in this non-pigmented no. 644 as a minor agglutinogen (no. 635) the no. 635 agglutinogen must be distinct from the more common *Staphylococcus aureus*, which does not agglutinate to any extent in no. 644 antiserum. Therefore it is reasonable to suppose that our pigmented, mannitol fermenting *Staphylococcus aureus* has been dissociated from a non-pigmented non-mannitol fermenting wild type, differing from no. 644 only in the kinds of pigmented agglutinogens that it contains.

Since the former are more virulent it is conceivable that the adaptation to the host was the result of such a dissociation, as we have shown experimentally with the alkaligenes-colon group (Study III—Mellon, 1926). Viewed in this way strain 744 can be looked on as an intermediate or perhaps a transition form which, by virtue of its failure to ferment mannitol and to yield pigment, is not yet the fully developed aureus indicated by its serological characters. There is no good reason for trying to make a separate group of such *apparently* anomalous strains, nor yet for saying that they do not fall into the group typified by strain no. 635.

THE GALL-BLADDER COCCI

Of rather special interest from the standpoint of the possible influence of environment in the transformations indicated is the similarity of the pigmented strains isolated from the gall-bladder wall or bile. This group, nos. 763, 3, 143 and 761, are lemon yellow, but mannitol negative, and by their agglutination with no. 644 serum show their relation to the aureus component of this coccus.

Table 3 clearly shows that the reaction of pigmented strain no. 761 with the serum no. 644 is with its aureus group, with which it forms a subgroup, since absorption of the no. 635 aureus serum removes the agglutinins specific for no. 761 and leaves those of no. 635. The agglutination of no. 761 with the no. 635 serum is then with minor agglutinins produced by the latter.

Strains no. 143 and 3 in their reactions indicate that strain no. 644 has another yellow component rather distinct from no. 635. The agglutination of these strains in no. 635 antiserum is slight or questionable, and when the no. 644 antiserum is ab-

TABLE 3

ANTIGEN	NO. 635				NO. 644				NO. 1				NORMAL			NaCl
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:20	1:40	1:80	
761	C C	3+ C	3+ C	C C	C C–	C C	C C	C C	C–	C–	C–	C–	0	0	0	00

	NO. 635A635				NO. 644A644				NO. 635A761				NO. 644A761			
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400
761	1–	0	0	0	C	C	3+	1+	0	0	0	0	0	0	0	0
635	0	0	0	0					C	C	C	C				
644					0	0	0	0					C	C	C	C

TABLE 4

ANTIGEN	NO. 635				NO. 644				NO. 1				NORMAL			NaCl
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:20	1:40	1:80	
3	1+	1+	0	0	C	C	C	C	C	C	C	C	2+	1+	0	00

TABLE 5

ANTIGEN	NO. 635				NO. 644				NO. 1				NORMAL			NaCl
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:20	1:40	1:80	
143	C C	C 2+	C 0	2+ 0	C C	C C	C C	C C–	2+	C	C	C	C	C	0	00

	NO. 635A635				NO. 635A644				NO. 644A644				NO. 644A635			
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400
143	0	0	0	0	C	C	C	C	0	0	0	0	C	C	C	1+
635	0	0	0	0	C	C	C	C	0	0	0	0	0	0	0	0

sorbed by the no. 635 aureus antigen the antibody corresponding to it is removed but not that corresponding to no. 143. Strain no. 763 is much like no. 143.

Here is a group of strains, some of which represent the dissociation of an additional pigmented group from that contained by the "common ancestor" white coccus. This group is distinct from the pigmented agglutinogen of no. 635. As was the case with no. 744 we have an incomplete strain of the type of no. 635, but the approach to it is a closer one because the latter are pigmented a light or lemon yellow even though they do not ferment mannitol. As is known, too, their virulence is apt to be intermediate.

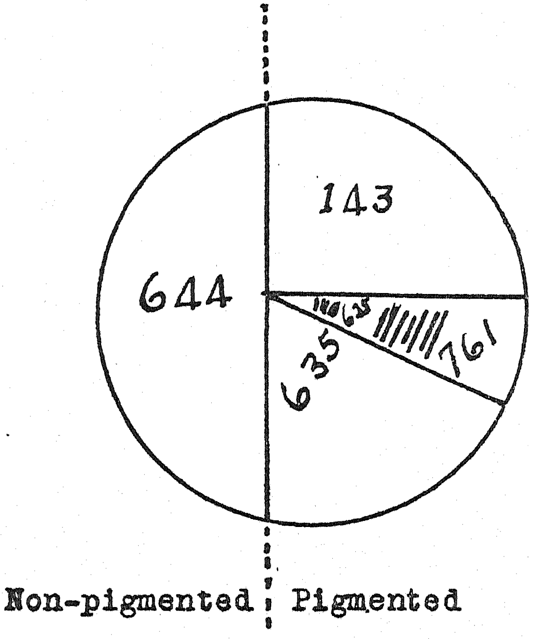

FIG. 1. The left hand sector represents the agglutinogen corresponding to *Staphylococcus albus* no. 644. Sector no. 635 represents a pigmented agglutinogen which contains at least one subgroup as shown by no. 761. Sector no. 143 represents a pigmented agglutinogen distinct from nos. 635 or 761.

So although, as has been shown in table 1, many strains show a correlation of pigment production and mannitol fermentation, others have not reached the type—so to speak—in either one or both particulars, so that the implications of the term *Staphylococcus aureus* and *S. albus* may be quite different with individual cocci unless they have been subjected to detailed study. What would be called the anomalous character of such strains, as regards fermentation, may be correlated with their origin from the bile since all pigmented strains isolated from this source were similar.

Diagramatically then, the common ancestor strain may be viewed as an agglutinogenic composite having at least the groups depicted in figure 1, and perhaps more, had we encountered the strains to bring them out.

On the basis of these considerations it becomes clear how dissociated strains within the animal body may not necessarily be related serologically and yet have come from the same source in an hereditary sense. On the other hand, they may play quite different rôles in the host, depending of course on their relative virulence.

OTHER SEROLOGICALLY DISTINCT STRAINS

While the majority of the aureus strains studied showed serologic relation with aureus strain no. 635, a few did not. Moreover, they showed no agglutination in the antiserum of no. 644, indicating that they were serologically distinct from the pigmented antigenic components of this strain. Thus we have encountered at least three distinct groups of aureus, to say nothing of subgroups. The strains distinct from no. 635 are nos. 628, 716, and 770-O. In addition to being pigmented these also ferment mannitol.

Similarly strains nos. 1030, 650 and 770-W, which are not pigmented and mannitol negative, do not react with the antiserum of strain no. 644. Strain no. 770-W came from the breast milk of the same case as no. 770-O. Not only are they quite distinct from the strains above considered but they are not related serologically to each other, as was shown by preparing antisera for each and doing cross agglutinations and

TABLE 6

Showing the agglutination results with the "white" variants of the pigmented cocci

ANTIGEN	PIGMENT	MANNITOL	NO. 635							NO. 644					NO. 635A635					NO. 644A635				NORMAL			NaCl	DATE
			1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:50	1:100	1:200	1:400	1:800	1:50	1:100	1:200	1:400	1:800	1:50	1:100	1:200	1:400	1:20	1:40	1:80		
635	Orange	+	C	C	C	C	C−	C−	0	C	C	C	C	0	1−	0	0	0	0	0	0	0	0	1−	0	0	00	5/8
635A	White	+	C	C	C	C	C	C−		C	C	3+	0	0	1+	1−	1−	0	0					C	1+	0	00	
597	Orange	+	C	C	C	1−	0	0		C	C	C−	0	0	0	0	0	0	0	0	0	0	0	0	0	0	00	
597A	White	+	C	C	C	C	C	1+		C	C	C	C	1+	0	0	0	0	0	0	0	0	0	0	0	0	00	
666	Orange	+	C	C	C	C	2+			C	C	C	0		0	0	0			0	0	0	0	1−	0	0	00	
666A	White	+	C	C	C	C	1+			C	C	C	1+											C	1+	0	00	
635	Orange	+	C	C	C	C	3+	1+	0																		00	5/12
635A	White	+	C	C	C	C	C−	1+	0																		00	
597	Orange	+	C	C	C	2+	1+	0	0																		00	
597A	White	+	C	C	C	C	C−	2+	1+																		00	

absorptions. It was thought at first that no. 770-W might be the non-pigmented form of no. 770-O.

In this connection it should be noted that the dissociation of non-pigmented strains from pigmented ones is by no means infrequent. Our no. 635 strain dissociated white colonies spontaneously on more than one occasion when the strain had been carefully purified. However, the greater number of these white colonies regain their orange color later, but the individual colonies vary greatly in this respect.

Inasmuch as strain no. 635 was used for developing a pure "yellow" antiserum it was desirable to be sure that the white colonies that it occasionally dissociated were not different antigenically. In fact they were always identical serologically as is clearly shown in table 6 by the agglutinations and absorptions. Moreover the "white" colonies always fermented mannitol.

RESULTS OF MANNITOL FERMENTATION

Despite the discrepancies in the reports as to the reliability of mannitol fermentation as a basis of differentiation, all of the cultures included in this series were tested in mannitol broth. Sugar free veal infusion broth, fermented with *B. coli*, and adjusted to a pH of 6.8 was used, plus bromothymol blue indicator after the method of Baker (1922). One per cent mannitol was added after sterilization. Cultures were incubated for seven days and readings made at one, three, five and seven days, respectively. As a rule, acid forming cultures had decolorized the green of the indicator to a yellow at the end of the third day. All acid forming cultures were further tested with methyl red. Readings were made after the method of Clark (1920) and no electrometric readings were taken.

All of the orange pigmented cultures fermented mannitol with the formation of acid but not of gas, reaching a pH of 6.0 to 5.4 at the end of seven days. All of the non-pigmented cultures, with one exception, *failed* to form either acid or gas in mannitol broth. Only one non-pigmented staphylococcus of this series fermented mannitol. Four yellowish cocci from gall-bladder material failed to ferment mannitol.

The results of the fermentation tests are summarized in table 7.

In view of the fact that our results with mannitol fermentation agree with those of Hine (1922) and are at variance with those of Winslow (1908), of Walker and Adkinson (1917) and of Julianelle, tests were repeated on a few of the cultures with the media of Julianelle (1922) and of Winslow, consisting of a 1 per cent pepton broth to which 1 per cent mannitol was added. The results are shown in table 8.

TABLE 7

Showing the results of the mannitol fermentation tests

COLOR	MANNITOL POSITIVE	MANNITOL NEGATIVE	TOTAL
Orange	33	0	33
White	1	20	21
Yellow	0	4	4
Total	34	24	58

TABLE 8

Showing the results in pH of further mannitol fermentation tests after seven days incubation

STRAIN	INFUSION BROTH	PEPTON BROTH
644	6.8	6.8
646	6.8	6.8
657-B	6.8	6.8
659	6.8	6.8
734	6.4	5.2
630	5.6	5.2
635	6.0	5.4
657	6.0	5.4

It is therefore apparent from table 7 that with the strains under consideration mannitol fermentation was in the main correlated with pigment formation. Upon the addition of mannitol, either sugar free infusion broth or pepton broth provided a suitable medium for differentiation.

For routine diagnostic work staphylococci that produced an orange pigment were reported as *Staphylococcus aureus* and non-pigmented cultures as *Staphylococcus "albus."* The aureus cultures sometimes showed good color in twenty-four hours, but in many cases several days passed before pigmentation was obvious. Upon plating, orange colonies and white colonies were sometimes obtained from a pigmented culture. Some of the white colonies so obtained remained colorless during a long series of subcultures but later became pigmented. Some of the chromogenic cultures lost and regained chromogenesis during the period that they were under observation. This is in accord with the observations of other workers who have found chromogenesis to be a variable function, easily influenced by environmental conditions.

A few of the gall-bladder cocci were yellowish in color and seemed to be of the citreus type. The majority of the cultures however, were definitely either orange or colorless. Practically all of the staphylococci studied showed definite hemolysis when planted on blood agar.

LITERATURE REVIEW AND DISCUSSION

The earliest attempts at classification appear to have been based largely upon differences in pigmentation. Kolle and Otto in 1902 first differentiated pathogenic from saprophytic forms by means of agglutination tests. Gordon (1905), after extensive work on the classification of the streptococci, undertook the classification of the staphylococci. His contributions in 1905 and 1906 deal with studies on several hundred staphylococci, "mainly white forms," and using liquefaction of gelatin, coagulation of milk and acidification of mannitol and glycerol as means for separation into four types.

Dudgeon (1908) found slightly more active fermentative powers associated with the production of orange pigment but considered the white and orange cocci varieties of a single species. The orange forms attacked mannitol, glycerol and raffinose actively. The Winslows, also in 1008, divided the cocci into two main groups, one essentially parasitic and active in fermentative powers (orange and white) and the other (yellow and red) saprophytes and with more restricted fermentative powers.

They further divided them on the basis of gelatin liquefaction associated with pigment production and considered the orange and the white forms as constituting different genera. Kligler (1913) substantiated the Winslows' basis of separation by means of the rate of liquefaction of gelatin.

Walker and Adkinson (1917), working with sera of rather low titer (C/250), found that aureus immune serum would agglutinate with aureus but not with albus strains, and that albus immune serum would agglutinate with albus but not with aureus strains. They used Hiss serum water for fermentation tests and found that while aureus always fermented mannitol, ten strains of albus were manitol-negative while five were mannitol-positive. Winslow, Rothberg and Parsons (1920) find that staphylococci from pathogenic sources tend to ferment more strongly then saprophytic, but concluded that there is no justification for a "generic distinction between the orange and the white staphylococci." They place mannitol among the carbohydrates "attacked so rarely as to be of no serious diagnostic value."

Julianelle (1922) has done intensive work on the serological and biochemical relations of 25 staphylococci, including four saprophytic forms, and divides them into three main groups and two subgroups on the basis of agglutination and absorption tests. But "these groups do not conform strictly with differences in pigment formation and other biological characters (Bergey)." Julianelle finds that mannitol is always fermented by his staphylococci, in fact finds no difference in fermentative faculties between his parasitic and saprophytic forms.

Hine (1922) classifies 81 pathogenic staphylococci by serological means into two main groups, the pyogenes and the epidermidis, and finds that in general the pyogenes group are pigmented and ferment mannitol while the epidermidis group in general are non-pigmented and do not ferment mannitol. By absorption tests he found further subgroups within his two main groups.

In Bergey's "Manual of Determinative Bacteriology" (1923) the staphylococci are classified first on the basis of pigment formation, orange pigmented being called *Staphylococcus aureus*, lemon yellow cocci *Staphylococcus citreus*, and the white forms *Staphylococcus epidermidis, albus*, etc. Aureus, citreus and albus are fermenters of mannitol while epidermidis is not. Bergey does not indicate the serological relationship of the various strains. Culture no. 39 would be *Staphylococcus albus* according to Bergey but serologically it is identical with *Staphylococcus aureus* and we have so classified it, regarding it as a non-pigmented aureus. *Staphylococcus citreus*, according to the "Manual," is a lemon yellow mannitol fermenter. Our lemon yellow cultures are all mannitol-negative and serologically belong to our type aureus, or are of a subgroup, or they may be a distinct antigenic aureus. Moreover these aureus groupings are components of the common ancestor strain no. 644.

Our results with mannitol fermentation are particularly interesting in view of the divergent reports of other bacteriologists. Winslow and Rothberg report that the staphylococci are generally mannitol-negative; Julianelle reports them invariably mannitol-positive; Walker and Adkinson found that while pigmentation and agglutination were correlated and aureus always fermented mannitol, *Staphylococcus albus* (Epidermidis?) was mannitol-positive in a few cases and mannitol-negative in others. Hine on the other hand found pigmentation, mannitol fermentation and serological affinities to be closely correlated and most of our strains happen to confirm this correlation in a general way.

Although pigment formation and mannitol fermentation taken together served to allocate in a general way the majority of the strains with which we worked, with another series of strains these characters might not be so successful. Indeed as we indicate they are by no means final criteria. Then, too, a mannitol-negative, non-pigmented strain may not be an epidermidis strain in the sense of being serologically distinct from the aureus strain as nos. 644 and 1 show clearly.

Although as we have already remarked, our primary object was not to set up a classification of staphylococci, the probable lines of descent indicated by the common ancestor strain possessed of component groups—both as to antigenic constitution and pigment formation—help us to see the characters of the group in somewhat better perspective. The results seem to indicate that on a broad biologic basis it is not feasible to consider but one character in classification, however desirable it may be on practical grounds. The characters must be taken as a whole and with flexibility of limits, else inevitably we shall have a varying number of unclassifiable strains.

CONCLUSIONS

1. We have described a non-pigmented strain of staphylococcus which we regard as a probable common ancestor of distinct strains of *Staphylococcus aureus* and *S. albus*.
2. The aureus strains, which cross agglutinate and absorb from the antiserum of the non-pigmented common ancestor strain, represent a dissociation of a strain possessed of "specific," "pigmented" agglutinogen, potentially present in the parent non-pigmented strain.
3. This pure parent strain contains as its antigenic components at least two distinct "aureus" agglutinogens and at least one "albus" agglutinogen.
4. Explanation is offered for the genetic origin of many of the "atypical" strains, i.e., those where correlation of such characters as pigment production and mannitol fermentation fail.
5. The heterogeneity resulting from this situation renders classification especially difficult, if the resulting system is supposed to make for ready identification on the basis of a single set of characters.
6. Of the 58 strains studied relatively few of the apparently pure "aureus" agglutinogens were closely related to these groups present in the common ancestor strain. They may have originated from other similar ancestral strains with multiple agglutinogens. On the other hand, the majority of the albus strains were similar to the "white" agglutinogen of the "parent" strain.
7. It seems probable that this represents one of several wild or aboriginal saprophytic strains that become dissociated into

the more parasitic albus and aureus strains. This dissociation may be a mechanism of adaptation when a strain becomes pathogenic.

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A RAPID METHOD FOR THE DETERMINATION OF SUGAR IN BACTERIAL CULTURES[1]

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The fermentability or non-fermentability of a given sugar is a question which must be constantly decided in the classification of bacteria. Because of the number of sugars and often the larger number of cultures involved, the bacteriologist usually resorts to indirect methods to determine his decision. Turbidity of the media, gas or acid production are the usual criteria taken to indicate fermentation. Everyone realizes that these indirect methods are uncertain and often misleading. Some bacteria produce little or no cloudiness in the media, large numbers produce such small quantities of gas that it cannot be detected except by very refined methods, and others convert most of the sugar into neutral products, such as ethyl alcohol, mannitol, acetone, etc. (Stiles, Peterson and Fred, 1925; Reilly, Hickenbottom, Henley and Thaysen, 1920). In many instances fermentations are best made with an excess of $CaCO_3$ and in such cases gas and acid production are excluded as a means of determining fermentability. In a recent study of anaerobic bacteria Brown (1925) emphasizes the difficulty of determining the fermentability of sugars by indirect methods.

A direct method of measuring the sugar destroyed is much to be preferred. It gives a definite and accurate measure of the fermentability of the sugar. Such a method to be widely used must be quick and fairly accurate. The gravimetric methods

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give accurate results, but are too long and tedious. Colorimetric methods are rapid but they are unsuited to colored solutions and require clarification of the medium. Standards with equivalent amounts of sugar, which are difficult to prepare and hard to keep, must be used. Colorimetric sugar analysis also requires a high-class technique which is not always available.

Of the volumetric methods, Shaffer and Hartmann's (1920–1921) offers the best possibilities. It is rapid, accurate, and requires no filtration of the Cu_2O. A solution of 0.1 N thiosulphate is the only standard reagent required. In principle this method consists in oxidizing the cuprous oxide formed by the sugar with iodine, and titrating the excess of the latter with thiosulphate. The iodostarch end point obtained here is readily observable and is one of the most sensitive end points in volumetric analysis. The volumetric method of Shaffer and Hartmann is applicable to Fehling's solution, Benedict's reagent, and Folin's reagent. The latter, because it is easy to prepare, is permanent, and gives a maximum amount of cuprous oxide, is to be preferred for dilute sugar solutions. Shaffer and Hartmann's macro method using Fehling's solution will accommodate a sample containing from 3 to 100 mgm. of glucose. Their micro method is adapted to samples containing from 0.1 to 2 mgm. of reducing sugar, and is particularly suited to the determination of sugar residues in bacterial cultures.

METHODS OF ANALYSIS

1. Reagents

a. Combined micro reagent:

	grams per liter
$CuSO_4 \cdot 5H_2O$	5.0
Tartaric acid	7.5
Na_2CO_3 (anhydrous)	40.0
KI	10.0
KIO_3	0.7
Potassium oxalate	18.4

The sodium carbonate is dissolved in about 400 cc. of warm water, and into this, with stirring, is poured the copper sulphate and tartaric acid dissolved in about 150 cc. of water. The iodate, iodide, and oxalate are dissolved in about 250 cc. of water, rinsed into the alkaline copper solution, cooled and diluted to a liter. It is usually more convenient to make up a larger quantity based on the same proportion of reagents.

b. 1 N H_2SO_4—27 cc. of concentrated H_2SO_4 are poured into a quantity of water and diluted to a liter. The resulting solution is approximately normal but can be readily adjusted by titration.

c. 0.1 N thiosulphate—25 grams of pure sodium thiosulphate and 1 gram of NaOH are dissolved in water and diluted to a liter. This should give a solution a trifle stronger than 0.1 N. It can be readily standardized with a solution of 0.1 N $K_2Cr_2O_7$. To prepare this solution pure $K_2Cr_2O_7$ is dried at 110°C., and 4.9033 grams are dissolved in water and diluted to 1 liter. Twenty-five cubic centimeters of the standard dichromate are transferred to a large beaker containing about 3 grams KI and 10 cc. strong HCl in aqueous solution. The contents are diluted to 500 or 600 cc. and titrated with the thiosulphate. Starch paste is added toward the end of the reaction, and at the end point the solution turns from a blue to a light green. If the thiosulphate used be pure, the volume will be a little less than 25 cc., and the solution can be readily adjusted to 0.1 N by dilution with water. Thus if the titration is 24.7 cc. thiosulphate, every 24.7 cc. of thiosulphate should have added to it 0.3 cc. water, or to 914 cc. of the remaining solution add $\frac{914}{24.7} \times 0.3$ or 11.1 cc. of water. The resulting solution should be 0.1 N and can be readily checked against the 0.1 N dichromate. The 0.1 N thiosulphate thus prepared will keep its strength for more than a year. To make 0.005 N thiosulphate, 25 cc. of the 0.1 N solution are diluted to 500 cc. in a volumetric flask and mixed. This solution keeps for only a few days and is best prepared anew for each set of determinations.

d. Basic lead acetate, Horne reagent—A 33 per cent solution is used.

e. Phosphate solution—For removing excess lead, a 10 per cent solution of $Na_2HPO_4 \cdot 12H_2O$ is used. Three cubic centimeters are required for every cubic centimeter of the lead acetate solution used. Add phenolphthalein and if alkaline or acid neutralize.

2. Preparation of the sample for analysis

The usual practice is to clarify the solution by adding the basic lead acetate, filtering and removing the excess lead with sodium carbonate or other lead precipitant. A number of deleading agents have been tried and of these disodium phosphate has been found the most satisfactory. The precipitate of lead phosphate settles in less than three minutes and an aliquot of the clear supernatant liquid can be pipetted off for analysis. Instead of filtering off the lead proteinate and removing the excess lead from the filtrate these two steps may be combined in one operation without introducing an appreciable error.

Place 10 or 25 cc. of culture, depending on the percentage of sugar, in a 50 cc. volumetric flask, add a few drops of phenolphthalein and neutralize with sodium hydroxide. Add 1 cc. of lead acetate solution, shake, and then add 3 cc. of phosphate solution. If alkaline or acid, neutralize, dilute to exactly 50 cc. and mix thoroughly by inverting. Let stand for three minutes and then remove an aliquot for analysis by means of a pipette. If chlorides or other compounds precipitable by lead are present, a little more of the lead acetate solution can be used. An excess of lead acetate is to be avoided for some of the sugar will be carried down with the precipitate.

3. Determination

Place 5 cc. of the micro reagent in a 50 cc. Pyrex test tube and to this add from 1 to 5 cc., depending upon the quantity of sugar present, of the clarified sample. If less than 5 cc. of sample is taken, add sufficient water to make the total volume 10 cc. At the same time make up a blank with 5 cc. of water and 5 cc. of reagent. Stopper the test tubes with loose-fitting corks, to prevent oxidation from the air, and heat for 15 minutes in a boiling water bath. Cool in running water, add 5 cc. of 1 N

H_2SO_4, shake well, let stand one minute and titrate with 0.005 N thiosulphate and starch paste as an indicator. Add the starch solution when the solution has turned a light straw color which indicates that only a trace of iodine remains. Continue the titration with thiosulphate until the blue color of the starch iodine compound completely disappears. The end point is very sharp—within 1 to 2 drops of thiosulphate.

For convenience in calculation, the authors have modified the table given by Shaffer and Hartmann so that it is possible to obtain milligrams of sugar direct from the difference in titration between the blank and the sample (see table 1). If an equivalent of 1 cc. of the original sample or a 1 cc. sample direct is taken, the calculations are lessened still more. By moving the decimal point 1 place to the left, the percentage of sugar is obtained directly.

Example

	0.005N thiosulphate
Titration of blank	19.0 cc.
Titration of sample	14.1 cc.
Difference in titration	4.9 cc.
Glucose represented by difference	0.729 mgm.
Sugar if 1 cc. sample was taken	0.073 per cent

When 1 cc. of culture is taken for the direct determination later described, this method will accommodate samples containing up to 0.20 per cent glucose. If the sugar percentage is greater, the sample must be diluted. For a sugar concentration of about 0.50 per cent, a convenient dilution would be 10 cc. made up to 50 cc. and a 1 cc. aliquot taken. If only a single sample is to be analyzed or a qualitative test is to be made, the sample may be boiled for two and one-half minutes over a micro burner. Shaffer and Hartmann give a special table for this procedure. The table published with this paper can be used, but the results will be somewhat low.

When larger concentrations of sugar are to be analyzed it is better to use Shaffer and Hartmann's macro method. To shorten the calculations in the macro procedure, we have computed table 2 from Munson and Walker's (Browne, 1912) table

TABLE I

Micro sugar table—glucose corresponding to difference in titration between control and sample

0.005 N THIOSUL-PHATE	GLUCSOE	0.005 N THIOSUL-PHATE	GLUCOSE	0.005 N THIOSUL-PHATE	GLUCOSE	0.005 N THIOSUL-PHATE	GLUCOSE
cc.	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*
0.3	0.067	4.1	0.622	8.1	1.159	12.1	1.649
0.4	0.086	4.2	0.634	8.2	1.173	12.2	1.662
0.5	0.105	4.3	0.647	8.3	1.186	12.3	1.674
0.6	0.125	4.4	0.660	8.4	1.198	12.4	1.687
0.7	0.142	4.5	0.672	8.5	1.211	12.5	1.700
0.8	0.157	4.6	0.685	8.6	1.224	12.6	1.713
0.9	0.173	4.7	0.698	8.7	1.237	12.7	1.728
1.0	0.191	4.8	0.713	8.8	1.249	12.8	1.742
		4.9	0.729	8.9	1.262	12.9	1.756
1.1	0.210	5.0	0.745	9.0	1.275	13.0	1.770
1.2	0.229						
1.3	0.247	5.1	0.759	9.1	1.288	13.1	1.785
1.4	0.263	5.2	0.772	9.2	1.300	13.2	1.800
1.5	0.279	5.3	0.784	9.3	1.313	13.3	1.813
1.6	0.294	5.4	0.797	9.4	1.326	13.4	1.827
1.7	0.306	5.5	0.810	9.5	1.339	13.5	1.842
1.8	0.319	5.6	0.822	9.6	1.354	13.6	1.856
1.9	0.332	5.7	0.837	9.7	1.368	13.7	1.871
2.0	0.344	5.8	0.852	9.8	1.382	13.8	1.885
		5.9	0.868	9.9	1.397	13.9	1.899
2.1	0.357	6.0	0.882	10.0	1.411	14.0	1.913
2.2	0.370						
2.3	0.382	6.1	0.892	10.1	1.424	14.1	1.928
2.4	0.395	6.2	0.902	10.2	1.435	14.2	1.942
2.5	0.408	6.3	0.911	10.3	1.446	14.3	1.956
2.6	0.421	6.4	0.926	10.4	1.457	14.4	1.971
2.7	0.434	6.5	0.940	10.5	1.469	14.5	1.984
2.8	0.446	6.6	0.955	10.6	1.480	14.6	1.997
2.9	0.461	6.7	0.969	10.7	1.491	14.7	2.010
3.0	0.477	6.8	0.983	10.8	1.502	14.8	2.022
		6.9	0.997	10.9	1.513	14.9	2.035
3.1	0.493	7.0	1.010	11.0	1.524	15.0	2.048
3.2	0.507						
3.3	0.520	7.1	1.023	11.1	1.535		
3.4	0.532	7.2	1.036	11.2	1.547		
3.5	0.545	7.3	1.048	11.3	1.558		
3.6	0.558	7.4	1.061	11.4	1.569		
3.7	0.571	7.5	1.074	11.5	1.580		
3.8	0.583	7.6	1.088	11.6	1.591		
3.9	0.596	7.7	1.102	11.7	1.602		
4.0	0.609	7.8	1.116	11.8	1.613		
		7.9	1.130	11.9	1.624		
		8.0	1.145	12.0	1.636		

TABLE 2

Macro sugar table—glucose corresponding to difference in titration between control and sample

0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE
cc.	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*
1.1	3.1	5.1	15.4	9.1	27.9	13.1	40.5
1.2	3.4	5.2	15.7	9.2	28.2	13.2	40.9
1.3	3.7	5.3	16.1	9.3	28.5	13.3	41.2
1.4	4.0	5.4	16.4	9.4	28.9	13.4	41.5
1.5	4.3	5.5	16.7	9.5	29.2	13.5	41.8
1.6	4.6	5.6	17.0	9.6	29.5	13.6	42.1
1.7	5.0	5.7	17.3	9.7	29.8	13.7	42.4
1.8	5.3	5.8	17.6	9.8	30.1	13.8	42.8
1.9	5.6	5.9	17.9	9.9	30.4	13.9	43.1
2.0	5.9	6.0	18.2	10.0	30.7	14.0	43.4
2.1	6.2	6.1	18.5	10.1	31.1	14.1	43.8
2.2	6.5	6.2	18.8	10.2	31.4	14.2	44.1
2.3	6.8	6.3	19.1	10.3	31.7	14.3	44.4
2.4	7.1	6.4	19.5	10.4	32.0	14.4	44.7
2.5	7.4	6.5	19.8	10.5	32.3	14.5	45.0
2.6	7.7	6.6	20.1	10.6	32.6	14.6	45.3
2.7	8.0	6.7	20.4	10.7	32.9	14.7	45.6
2.8	8.3	6.8	20.8	10.8	33.3	14.8	46.0
2.9	8.6	6.9	21.1	10.9	33.6	14.9	46.3
3.0	8.9	7.0	21.4	11.0	33.9	15.0	46.6
3.1	9.3	7.1	21.7	11.1	34.2	15.1	46.9
3.2	9.6	7.2	22.0	11.2	34.5	15.2	47.2
3.3	9.9	7.3	22.3	11.3	34.9	15.3	47.6
3.4	10.2	7.4	22.6	11.4	35.2	15.4	47.9
3.5	10.5	7.5	22.9	11.5	35.5	15.5	48.2
3.6	10.8	7.6	23.2	11.6	35.8	15.6	48.5
3.7	11.1	7.7	23.5	11.7	36.1	15.7	48.9
3.8	11.4	7.8	23.8	11.8	36.5	15.8	49.2
3.9	11.7	7.9	24.1	11.9	36.8	15.9	49.5
4.0	12.0	8.0	24.5	12.0	37.1	16.0	49.8
4.1	12.3	8.1	24.8	12.1	37.4	16.1	50.1
4.2	12.7	8.2	25.1	12.2	37.7	16.2	50.5
4.3	13.0	8.3	25.4	12.3	38.0	16.3	50.8
4.4	13.3	8.4	25.7	12.4	38.3	16.4	51.1
4.5	13.6	8.5	26.0	12.5	38.7	16.5	51.5
4.6	13.9	8.6	26.3	12.6	39.0	16.6	51.8
4.7	14.2	8.7	26.6	12.7	39.3	16.7	52.1
4.8	14.5	8.8	27.0	12.8	39.6	16.8	52.4
4.9	14.8	8.9	27.3	12.9	39.9	16.9	52.7
5.0	15.1	9.0	27.6	13.0	40.2	17.0	53.0

TABLE 2—*Continued*

0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE	0.1 N THIOSULPHATE	GLUCOSE
cc.	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*
17.1	53.4	21.1	66.4	25.1	79.6	29.1	92.9
17.2	53.7	21.2	66.7	25.2	79.9	29.2	93.3
17.3	54.1	21.3	67.0	25.3	80.2	29.3	93.6
17.4	54.4	21.4	67.3	25.4	80.5	29.4	94.0
17.5	54.7	21.5	67.7	25.5	80.9	29.5	94.3
17.6	55.0	21.6	68.0	25.6	81.2	29.6	94.6
17.7	55.3	21.7	68.3	25.7	81.5	29.7	94.9
17.8	55.6	21.8	68.6	25.8	81.9	29.8	95.3
17.9	56.0	21.9	69.0	25.9	82.2	29.9	95.6
18.0	56.3	22.0	69.3	26.0	82.6	30.0	96.0
18.1	56.6	22.1	69.6	26.1	82.9	30.1	96.3
18.2	56.9	22.2	70.0	26.2	83.2	30.2	96.6
18.3	57.2	22.3	70.3	26.3	83.6	30.3	97.0
18.4	57.5	22.4	70.6	26.4	83.9	30.4	97.3
18.5	57.9	22.5	71.0	26.5	84.2	30.5	97.7
18.6	58.2	22.6	71.3	26.6	84.5	30.6	98.0
18.7	58.6	22.7	71.6	26.7	84.9	30.7	98.3
18.8	58.9	22.8	71.9	26.8	85.3	30.8	98.7
18.9	59.2	22.9	72.3	26.9	85.6	30.9	99.0
19.0	59.5	23.0	72.6	27.0	85.9	31.0	99.4
19.1	59.9	23.1	72.9	27.1	86.2		
19.2	60.2	23.2	73.2	27.2	86.6		
19.3	60.5	23.3	73.6	27.3	86.9		
19.4	60.8	23.4	73.9	27.4	87.2		
19.5	61.1	23.5	74.2	27.5	87.5		
19.6	61.5	23.6	74.6	27.6	87.9		
19.7	61.8	23.7	75.0	27.7	88.2		
19.8	62.1	23.8	75.3	27.8	88.5		
19.9	62.5	23.9	75.6	27.9	88.9		
20.0	62.8	24.0	75.9	28.0	89.2		
20.1	63.1	24.1	76.2	28.1	89.6		
20.2	63.4	24.2	76.5	28.2	89.9		
20.3	63.7	24.3	76.9	28.3	90.2		
20.4	64.0	24.4	77.2	28.4	90.6		
20.5	64.4	24.5	77.6	28.5	90.9		
20.6	64.7	24.6	77.9	28.6	91.3		
20.7	65.1	24.7	78.2	28.7	91.6		
20.8	65.4	24.8	78.6	28.8	91.9		
20.9	65.7	24.9	78.9	28.9	92.2		
21.0	66.1	25.0	79.2	29.0	92.6		

so that milligrams of glucose can be read directly from the difference in titration between the control and the sample.

The micro method is much to be preferred if a number of sugar determinations are to be made on fermented cultures. Less time is required, and the cost of materials per sample in the micro procedure is much less.

EXPERIMENTAL PART

The accuracy of the micro method and its suitability for the estimation of the principal sugars used in bacteriological work was determined by analyzing various culture media and comparing the results with those obtained by the macro method. Also, three different methods of clarification were tried and the results compared both with one another and with the figure found when no clarification was employed. In table 3 these methods are numbered 1, 2, 3, 4 and consisted of the following procedures:

In procedure 1 the sample was neutralized, clarified with basic lead acetate, filtered and an aliquot deleaded by adding 20 per cent Na_2CO_3 until slightly alkaline to phenolphthaline. After being made up to 50 cc., the $PbCO_3$ was filtered off and a portion taken for analysis. In no. 2 the treatment was the same as for no. 1, except that $Na_2HPO_4 \cdot 12H_2O$ was used as the deleading agent. Three cubic centimeters of phosphate solution were added for each cubic centimeter of lead acetate used. In the third modification the sample was clarified and deleaded in one operation. Sodium phosphate was used as the deleading agent. In the fourth method the sample was analyzed directly with no neutralization or clarification.

Procedure 1 is the method generally employed. The results of procedure 2, however, agree very well with those of the standard method. It has the advantage that the precipitate of lead phosphate settles much more rapidly than the lead carbonate. The results obtained by procedure 3 are not appreciably different from those obtained by the first two methods, and the saving in time is considerable. Procedure 4, or the direct method, gave in all but one case acceptable results. In the majority of cases where extremely accurate results are not required this method of direct analysis can be used to advantage. However, clarification by procedure 3 is rapidly executed and gives assurance of accurate results.

TABLE 3

Comparison of the macro and micro methods of sugar analysis as applied to various media and to various methods of clarification

ORGANISM	MEDIA	METHOD	UNFERMENTED SUGAR CALCULATED AS GLUCOSE			
			Method of clarification			
			1	2	3	4
			per cent	*per cent*	*per cent*	*per cent*
S. lactis..............	Yeast water	Macro	1.10	1.11	1.10	1.13
		Micro	1.15	1.17	1.22	1.21
	Yeast water plus $CaCO_3$	Macro	0.08	0.08	0.09	0.08
		Micro	0.09	0.09	0.10	0.10
B. coli...............	Beef-extract broth	Macro	0.79	0.79	0.80	0.68
		Micro	0.81	0.82	0.83	0.84
	Beef-extract broth and $CaCO_3$	Macro	0.20	0.21	0.19	0.18
		Micro	0.21	0.21	0.22	0.22
	"	Macro	0.05	0.05	0.06	0.06
		Micro	0.05	0.05	0.06	0.06
Acetone butyl alcohol..	Pepton	Macro			0.63	
		Micro			0.65	
Silage flora...........	Silage juice	Macro			0.15	
		Micro			0.14	
Yeast.................	Raisin extract	Macro	3.76	3.86	3.88	3.90
		Micro	4.05	4.00	4.09	4.15

In table 3 are given the data comparing the micro with the macro method in 26 samples whose glucose content ranged from 0.05 to 4.15 per cent. The agreement between the two methods is within 5 per cent in most instances and in many cases the figures check exactly. The micro method is particularly adapted to cultures having a low concentration of sugar. For samples containing over 1 per cent sugar the macro method, because of its wider range, is to be preferred.

In a study of the fermentability of sugar, an amount of carbohydrate must be taken sufficiently large to provide a range of sugar destruction and at the same time be well beyond the error of analysis. If the micro method is employed to determine residual sugar, culture media containing 0.5 per cent of sugar would be convenient. If unfermented a residue of 0.4 to 0.5 per cent sugar would be found, and if the fermentation is complete, the percentage would be less than 0.05. Amounts of sugar required for an initial concentration of 0.5 per cent are not so excessive as to limit the use of rare sugars, and the range of concentration from 0.5 to 0.05 per cent is sufficient to determine the fermentability or non-fermentability of any sugar.

TABLE 4

Factors for calculating the quantity of other sugars from the value for glucose

SUGAR	FACTORS CALCULATED FROM BROWN'S SUGAR ANALYSIS	FACTORS DETERMINED BY MICRO METHOD
Arabinose	0.97	1.06
Xylose	1.02	0.96
Galactose	1.11	1.14
Fructose	1.09	1.04
Maltose	1.64	1.79
Lactose	1.42	1.65
Sucrose (invert sugar)	1.04	1.02*

* Computed value.

Another advantage in the use of low concentrations of sugar is that only moderate acidities are developed. In such cases if the medium does not contain much protein the sugar content may be determined directly without neutralization or clarification. This method gives fair accuracy and is a great saving in time.

A further test of the accuracy of the two methods was made by adding known amounts of glucose to a culture medium and determining the increase of sugar. The agreement between the calculated and observed values varied from 0.01 to 0.04 per cent for the macro method. Recovery by the micro method was fully as good or better than by the macro method.

When sugars other than glucose are employed in the culture media a factor must be used in calculating the true sugar content. This is due to the fact that these sugars do not have the same reducing power as glucose. The ratio to glucose varies, not only with the kind of sugar, but also with the composition of the copper reagent. With the exception of maltose and lactose the reducing power of each of the other sugars is so nearly equal to that of glucose that in most bacteriological work the differences may be neglected. Table 4 gives the factors to be used for more accurate work. The figures given for the macro method were computed from Brown's Handbook of Sugar Analysis. The use of these factors may be better illustrated by an example showing the calculation of the maltose content of a culture medium.

	milligrams of sugar
Value found for glucose	1.48
Maltose in sample, 1.79 times 1.48	2.65

Sucrose must be hydrolyzed to invert sugar for its determination. For this inversion 1 cc. of concentrated HCl is added to 15 cc. of the sample and the mixture heated at 70°C. for ten minutes. After cooling, the solution is neutralized with NaOH.

SUMMARY

The Shaffer-Hartmann micro method has been adapted to the determination of 8 sugars commonly used in culture media. The method is rapid and accurate. The culture is neutralized, clarified with lead acetate and deleaded with sodium phosphate. The clarification and deleading may be combined in one operation effecting a gain in time and not appreciably lessening the accuracy of the determination. The solution clears in 3 minutes and the aliquot may be removed directly with a pipette. When the fermentability of the sugar is the only question involved, a determination may in most cases be made directly on the culture without neutralization or clarification.

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